CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450, on the date below:

November 28, 2005
Date

David L. Parker

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Bob G. Sanders *et al.*

Serial No.: 10/695,275

Filed: October 28, 2003

For: Tocopherols, Tocotrienols, Other Chroman and Side Chain Derivatives and Uses Thereof

Group Art Unit: 1623

Examiner: Khare, Devesh

Atty. Dkt. No.: D6150/CLFR:178USD1

DECLARATION OF BOB G. SANDERS, PH.D. AND KIMBERLY KLINE PH.D., UNDER 37 C.F.R. §1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

We, Bob G. Sanders, Ph.D. and Kimberly Kline, Ph.D., do declare that:

1. We are inventors of the subject matter disclosed and claimed in the above referenced patent application. As evidenced by the following, we both have recognized expertise in the field of cancer and cancer prevention and we have both published extensively on this subject:

For Dr. Bob G. Sanders:

- I have been a Professor at the University of Texas (U.T.) at Austin since 1973, and have been a member of the faculty there since 1968.
- I served as Associate Dean of Research and Development for the College of Natural Sciences at U.T. Austin for 10 years.
- I have published over 100 articles in peer-reviewed journals.
- I have served on a number of scientific advisory boards and review panels.
- In particular I have been a member if the American Institute for Cancer Research, Grant Review Panel, since 2000.
- A copy of my *curriculum vitae* which verifies the foregoing is attached as Exhibit A.

For Dr. Kimberly Kline:

- I have been a Professor at the University of Texas Austin since 1998 and have been a faculty member there since 1986.
- I have published over 70 peer-reviewed articles.
- I have served on a number scientific committees and review sections.
- I have been an Associate Editor for the *Journal of Nutrition and Cancer* since 1997.
- I have been a panel member for the American Institute for Cancer Research since 1996.
- A copy of my *curriculum vitae* which verifies the foregoing is attached as Exhibit B.

2. We understand that the Patent Examiner has alleged that the subject patent application does not enable a method for treating an individual with the group of chroman ring compounds that are claimed. Given our extensive expertise in this particular field we respectfully disagree with this opinion and assert that the specification provides ample teaching as to the effectiveness of these compounds and the methods of their use in the treatment of cell proliferative diseases.

3. The experiments described in the patent application assessed the anti-proliferative and apoptosis inducing activity of a class of chroman ring compounds. We extensively tested a broad group of these compounds in tissue systems as described in Example 7 and Tables 2 – 3 (pages 95 – 98 of the application). We found that none of the compounds was toxic to normal human mammary epithelial cells (see Tables 2 – 3 on pages 95 – 98 in the row labeled HMEC). Likewise none of the compounds induced apoptosis in immortalized, but non-tumorigenic cells, such as MCF-10A mammary cells (see Tables 2 – 3 on pages 95 – 98 in the row labeled MCF-10A). On the other hand, a variety of the chroman compounds were able to induce apoptosis in one or more of the tumor cell lines that we tested (see Tables 2 – 3 on pages 95 – 98, the numerical value indicates the concentration of the compound required to induce apoptosis in 50% of the treated cells). These studies indicated that a broad class of chroman ring compounds, comprising a variety of R groups were effective for arresting growth and inducing apoptosis in cancer cells, yet were not toxic to normal cells.

4. As indicated above, the experimental approach that we used for the *in vitro* screening assay enabled us to determine the effective concentration to induced apoptosis in 50% of the cells in culture (EC^{50}). Thus, we were able to compare the activities of the compound relative to one another (see the assay description on pages 92 – 94 and results in Tables 2 and 3). Using these methods, the effect of substituting individual groups at particular positions (R^1-R^5) on the chroman ring structure could be assessed. In the course of testing various compounds we found that an ethylenic group substituted at the R^5 position greatly enhanced the antiproliferative activity of the compounds. Experiments summarized in Table 1 on page 92 demonstrate that a variety of chroman compounds (*i.e.* α-, γ- and δ- tocotrienols) with an ethylenic R^5 group are as much as 40 times more potent inducers of apoptosis as compared to analogous compounds with a saturated R^5 group (see Figure 1 for the structures of α-, γ- and δ- tocotrienols and tocopherols).

These studies indicated that substitution of the ethylenic R^5 group increased the activity of the chroman compounds and that the increased activity was independent of other modifications of the chroman head, since the α-, γ- and δ- forms of the compound all demonstrated increased activity.

5. In parallel with the *in vitro* cell culture studies, we further validated effectiveness of the chroman ring compounds in studies using mouse model systems (see Example 15, page 109 – 112 for a description of the mouse model systems studied). These *in vivo* studies were undertaken using a model chroman ring compound, compound 1 (see page 25, lines 10 – 15), referred to as α-TEA. Data presented in Table 7, on page 120 of the specification, demonstrated that this compound was in fact able to reduce tumor weight gain as compared to the vehicle alone (compare tumor weight gain in rows labeled "#1" with those labeled "Vehicle"). Furthermore, the studies confirmed that the chroman compounds did not have appreciable toxicity, as indicated by the *in vitro* studies (see page 114, lines 3 – 10).

6. We have since conducted additional studies with α-TEA that further the support the effectiveness of the chroman ring compounds as therapeutic anti-proliferative agents. For example, we have shown that α-TEA can reduce the human mammary tumor burden in mouse model systems and prevent metastasis of these cancer cells (Lawson *et al.*, 2003, Exhibit C and Lawson et al., 2004a Exhibit D). Recently, we also determined that the antiproliferative activity of α-TEA can act synergistically with other chemotherapeutic compounds to prevent tumor growth (see Zhang *et al.*, 2004, Exhibit E and Lawson *et al.*, 2004b, Exhibit F). In each of these further *in vivo* studies the chroman ring compounds that tested were shown to be effective and nontoxic antiproliferative agents.

7. Given the established *in vivo* efficacy of the chroman ring compounds and the antiproliferative enhancing properties of the R^5 ethylenic group we synthesized compound 44,

which was identical to compound 1 (α-TEA), except for substitution of the R^5 ethylenic group (see page 88, lines 1 – 5). We found that the substitution of the R^5 ethylenic group also greatly enhanced the activity of this compound. This is shown in Table 4 on page 100, wherein the amount of compound 44 need to induce growth arrest in 50% of MDA-MB-43 tumor cells was shown to be 6 times less than the amount of compound 1 required for the same anti-proliferative activity. Furthermore, compound 44 was also 4 times more effective than compound 1 at inducing growth arrest in MCF-7 cells (Table 4 on page 100). We additionally assessed the ability of compound 44 to induce apoptosis in two cancer cells lines and found that is was superior to compound 1 in both cases (see Table 5 on page 100). Thus, we identified a particular subgroup of the chroman ring compounds (comprising an ethylenic R^5 group) that can be used as highly active antiproliferative therapeutics.

8. The specification also provides instruction as to how these compounds can be administered to an individual. We draw your attention to the detailed description of the invention, particularly pages 19 though 22, wherein methods of using the compounds that we have characterized in the treatment of an individual are set forth in some detail. These details include routes of administration (page 20 lines 5 – 8), methods administration (page 20 lines 12 – 16) and preferred dosage ranges (page 19, line 18 through page 20 line 4).

9. In conclusion, the foregoing demonstrates that the specification broadly enables the therapeutic use of the compounds wherein the R^5 group is ethylenic, in fact these compounds are the most effective compounds of the invention. We have shown, both *in vitro* and *in vivo*, that a range of chroman ring compounds are effective to induce growth arrest and apoptosis specifically in cancer cells. Furthermore, we have demonstrated that these compounds can be used *in vivo* to prevent cancer cell proliferation. Finally, we demonstrated that chroman ring compounds with an ethylenic group at the R^5 position have increased anti-proliferative efficacy,

and that this enhancement is independent of many other modification to the chroman compound (i.e. the enhancement is seen in α-, γ- and δ- tocotrienols as well as in compound 44). In view of the foregoing, it is evident that our patent specification provides the necessary instruction for one of skill in the art to practice the invention in an individual with the class of compounds that are claimed without undue experimentation.

10. We hereby declare that all statements made herein of our knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

11-1-05 Kimberly Kline

Date Kimberly Kline, Ph.D.

11-1-05 Bob G Sanders

Date Bob G. Sanders, Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Bob G. Sanders	Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Concord College, Athens, WV	B.S.	1954	Biology
Pennsylvania State University, University Park, PA	M.Ed.	1958	Biological Sciences
Pennsylvania State University, University Park, PA	Ph.D.	1961	Genetics
California Institute of Technology, Pasadena CA	Postdoc	1964-1966	Immunogenetics

A. Positions and Honors.

Positions and Employment

1957-1960 NSF Graduate Student Research Trainee Fellow, Pennsylvania State University with Dr. James E. Wright, Jr., University Park, Pennsylvania.
1960-1961 USPHS Pre-doctoral Fellow, Pennsylvania State University with Dr. James E. Wright, Jr., University Park, Pennsylvania.
1961-1964 Assistant Professor of Biology, Lafayette College, Easton, Pennsylvania.
1964-1966 USPHS Postdoctoral Fellow, California Institute of Technology with Dr. Ray D. Owen, Pasadena, California.
1966-1968 Assistant Professor, Department of Biology, California Institute of Technology, Pasadena, California.
1968-1973 Associate Professor, Department of Zoology, The University of Texas at Austin, Austin, Texas
1973-1999 Professor, Department of Zoology (until Biological Sciences reorganization), The University of Texas at Austin, Austin, Texas.
1999-present Professor, School of Biological Sciences, Section of Molecular Genetics and Microbiology, The University of Texas at Austin, Austin, Texas.

Other Experience and Honors

1963 Oak Ridge National Laboratory Summer Research Fellowship, Oak Ridge, Tennessee
1983 Texas Genetics Society, President
1984 University of Texas College of Natural Sciences Teaching Excellence Award
1985-1995 Associate Dean for Research and Development, College of Natural Sciences, The University of Texas at Austin, Austin, Texas.
1988-present Scientific Advisory Board for the University of Texas System Cancer Center-Science Park Research Division, member
2000-present American Institute for Cancer Research, Grant Review Panel II, Member
2003 Grant reviewer for the U.S. Department of Agriculture (USDA) Cooperative State Research, Education, and Extension Service (CSREES) Animal Health & Well Being. Spring 2003

B. Selected Peer-Reviewed Publications (in chronological order).

(Total of 104 peer-reviewed publications)

1. Yu, W., K. Israel, Q. Y. Liao, C. M. Aldaz, B. G. Sanders, and K. Kline. 1999. Vitamin E succinate VES) induces Fas sensitivity in human breast cancer cells: Role for Mr 43,000 Fas in VES- triggered apoptosis. *Cancer Research.* 59: 953-961.

2. Yu, W., M. Simmons-Menchaca, A. Gapor, B. G. Sanders, and K. Kline. 1999. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. *Nutrition and Cancer.* 33: 26-32.2. Herbert, B. S., B. G. Sanders, and K. Kline. 1999. N-(4-hydroxyphenyl) retinamide activation of transforming growth factor-β and induction of apoptosis in human breast cancer cells. *Nutrition and Cancer.* 34: 121-132.
3. Israel, K., W. Yu, B. G. Sanders, and K. Kline. 2000. Vitamin E succinate (VES) induces apoptosis in human prostate cancer cells: Role for Fas in VES-triggered apoptosis. *Nutrition and Cancer.* 36: 90-100.
4. Kline, K., W. Yu , and B. G. Sanders. 2001. Vitamin E: Mechanisms of action as tumor cell growth inhibitors. *Journal of Nutrition.* 131: 161S-163S.
5. Yu, W., Q. Y. Liao, F. M. Hantash, B. G. Sanders, and K. Kline. 2001. Activation of extracellular signal-regulated kinase and c-Jun-NH2-terminal kinase but not p38 mitogen-activated protein kinases is required for RRR-α-tocopheryl succinate-induced apoptosis of human breast cancer cells. *Cancer Research.* 61: 6569-6576.
6. You, H., W. Yu, B. G. Sanders, and K. Kline. 2001. RRR-α-tocopheryl succinate induces MDA-MB-435 and MCF-7 human breast cancer cells to undergo differentiation. *Cell Growth & Differentiation.* 12: 471-480.
7. You, H., W. Yu, D. Munoz-Medellin, P. H. Brown, B. G. Sanders, and K. Kline. 2002. Role of extracellular-regulated kinase pathway in RRR-α-tocopheryl succinate-induced differentiation of human MDA-MB-435 breast cancer cells. *Molecular Carcinogenesis.* 33: 228-236.
8. Yu, W., B. G. Sanders, and K. Kline. 2002. RRR-α-tocopheryl succinate-induction of DNA synthesis arrest of human MDA-MB-435 cells involves TGF-β independent activation of p21 (Waf1/Cip1). *Nutrition and Cancer.* 43: 227-236.
9. Lawson, K.A., K. Anderson, M. Menchaca, J. Atkinson, L-Z. Sun, V. Knight, B. E. Gilbert, C. Conti, B. G. Sanders, and K. Kline. 2003. Novel vitamin E analog decreases syngeneic mouse mammary tumor burden and reduces lung metastasis. *Molecular Cancer Therapeutics.* 2: 437-444.
10. Yu, W., B. G. Sanders and K. Kline. 2003. RRR-α-tocopheryl succinate-induced apoptosis of human breast cancer cells involves Bax translocation to mitochondria. *Cancer* Research. 61: 2483-2491.
11. Kline, K., K. A. Lawson, W. Yu, and B. G. Sanders. 2003. Vitamin E and breast cancer prevention: Current status and future potential. *Journal of Mammary Gland Biology and Neoplasia.* 8: 91-102.
12. Shun, M-C., W. Yu, A. Gapor, R. Parsons, J. Atkinson, B. G. Sanders, and K. Kline. 2004. Pro-apoptotic mechanisms of action of a novel vitamin E analog (α-TEA) and a naturally occurring form of vitamin E (δ-tocotrienol) in MDA-MB-435 human breast cancer cells. *Nutrition and Cancer.* 48: 95-105.
13. Anderson, K., M. Simmons-Menchaca, K. A. Lawson, J. Atkinson, B. G. Sanders and K. Kline. 2004. Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E succinate and vitamin E analogue, α-TEA. *Cancer Research.* 64: 4263-4269.
14. Lawson, K. A., K. Anderson, R. M. Snyder, M. Simmons-Menchaca, J. Atkinson, L-Z. Sun, A. Bandyopadhyay, V. Knight, B. E. Gilbert, B. B. Sanders and K. Kline. 2004. Novel vitamin E analogue and 9-nitro-camptothecin administered as liposome aerosols decrease syngeneic mouse mammary tumor burden and inhibit metastasis. *Cancer Chemotherapy and Pharmacology.* In Press.
15. Zhang, S., K. A. Lawson, M. Simmons-Menchaca, L-Z. Sun, B. G. Sanders, and K. Kline. 2004. Vitamin E analog α-TEA and celecoxib alone and together reduce human MDA-MB-435-FL-GFP breast cancer burden and metastasis in nude mice. *Breast Cancer Research and Treatment.* 87: 111-121.
16. Lawson, K. A., K. Anderson, M. Simmons-Menchaca, J. Atkinson, L-Z. Sun, B. G. Sanders, and K. Kline. 2004. Comparison of vitamin E derivatives α-TEA and VES in reduction of mouse mammary tumor burden and metastasis. *Experimental Biology and Medicine.* 229: 954-963.
17. Anderson, K., K. A. Lawson, M. Simmons-Menchaca, L-Z. Sun, B. G. Sanders and K. Kline. 2004. α-TEA plus cisplatin reduces human cisplatin-resistant ovarian cancer cell tumor burden and metastasis. *Experimental Biology and Medicine.* 229: 1169-1176.

C. Research Support.

Ongoing Research Support

Natural and Synthetic Agents for Treatment of Cancer
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: Foundation for Research
Award Period: January 1, 2005 - December 31, 2005
The goals of this project are to identify and characterize analogs of vitamin E that have anti-cancer properties.
Role: Co-Principal Investigator

Human Breast Cancer Cell Growth Inhibition by Vitamin E
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: DHHS-National Institutes of Health-National Cancer Institute
Award Period: August 1, 2005 – May 31, 2009
The goals of this project are to investigate the ability of alpha-TEA to be an effective chemotherapeutic agent for breast cancer alone and in combination with doxorubicin and to identify and characterized dysregulated signaling pathways/events in human breast cancer cells that are modified by alpha-TEA.
Role: Co-Principal Investigator

CORE GRANT
Title: "Mechanisms and Prevention of Environmental Disease"
Director: John DiGiovanni
Funding Agency: DHHS, PHS, NIH, Environmental Health Sciences Core Center Grant
Grant Number: ES07784
Total Award Period: April 1, 2001 through March 31, 2006

There are 26 faculty members from MD Anderson-Science Park Research Division; 13 faculty members from the University of Texas at Austin; and 13 faculty members from MD Anderson-Houston on this large Center Grant.

This grant provides money for the establishment and maintenance of shared service cores: Core 1: Molecular Biology; Core 2: Transgenic; Core 3: Histology & Tissue Proc.; Core 4: Flow Cytometry; Core 5: Analytical; and Core 6: Biostatistics.
Members of this core grant receive a discount in fees assessed for the use of these special research services.
Role: Member of Research Core 1 (Mechanisms of Toxicity and Cell Death).

On a competitive basis we were awarded a Pilot Project from the ES07784 core grant.
Title: "α-TEA & Celecoxib in Skin Cancer Prevention"
Participants: Kimberly Kline, Bob G. Sanders and Susan Fischer
Funding Agency: 5 P30 ES07784
Total Award Period: July 1, 2004 through June 30, 2005
The goals of this project are to assess the efficacy of a novel vitamin E analog and celecoxib alone and in combination to prevent UV-B induced skin cancer in the SKH hairless mouse model.
Role: Co-Investigator

TRAINING GRANTS
Title: NIEHS Toxicology Training Grant: "Training in Molecular Toxicology and Environmental Disease"
Director: John Richburg
Participants: Mark Bedford, Claudio Conti, Kevin Dalby, John DiGiovanni, Susan Fischer, Robin Fuchs-Young, David Johnson, James Kehrer, Kimberly Kline, Michael MacLeod, Ellen Richie, Bob Sanders, Dean Tang, Karen Vasquez, and Cheryl Walker
Funding Agency: National Institutes of Health / National Institute for Environmental Health Sciences

Total Award Period: July 1, 2003 through June 30, 2008
This is a training grant for support of eight pre-doctoral trainees. Currently Rachel Snyder, pre-doctoral student in the lab, holds a fellowship awarded by this training grant.
Role: Member

Title: "Short Term Research Training for Minority Students"
Director: John Richburg
Participants: Mark Bedford, Claudio Conti, Kevin Dalby, John DiGiovanni, Susan Fischer, Robin Fuchs-Young, David Johnson, James Kehrer, Kimberly Kline, Michael MacLeod, Ellen Richie, Bob Sanders, Dean Tang, Karen Vasquez, and Cheryl Walker
Funding Agency: National Institutes of Health / National Institute for Environmental Health Sciences
Total Award Period: April, 1, 2002 through March 31, 2007
Role: Member

Pending Research Support

Pilot Project from the ES07784 core grant.
Title: "α-TEA & Selenium in Prostate Cancer Prevention"
Participants: Bob G. Sanders, Claudio Conti and Kimberly Kline
Funding Agency: 5 P30 ES07784
Total Award Period: September 1, 2005 through August 31, 2006
The goals of this project are to assess the efficacy of a novel vitamin E analog and selenium alone and in combination to prevent prostate cancer in the TRAMP mouse model.
Role: Co-Principal Investigator

Completed Research Support

Natural and Synthetic Agents for Treatment of Cancer
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: Foundation for Research
Award Period: January 1, 2004 - December 31, 2004
The goals of this project are to identify and characterize analogs of vitamin E that have anti-cancer properties.
Role: Co-Principal Investigator

Human Breast Cancer Cell Growth Inhibition by Vitamin E
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: DHHS-National Institutes of Health-National Cancer Institute
Award Period: August 1, 2000 - July 31, 2005
The goals of this project are to identify and characterize the biochemical and cellular events involved in how vitamin E based compounds induced DNA synthesis arrest, cellular differentiation and apoptosis in human breast cancer cells.
Role: Co-Principal Investigator

Dietary Interactions to Inhibit Mammary Cancer
Participants: Kimberly Kline and Bob G. Sanders
Funding Agency: The State of Texas Coordinating Board ATP
Total Award Period: January 1, 2002 through December 31, 2003 (extended to August 31, 2004)
The goals of this project are to test whether three nutrient-based compounds singly or in combination exhibit enhanced chemotherapeutic and/or chemopreventive properties in different experimental models of breast cancer.
Role: Co-Principal Investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Kline, Kimberly	Professor of Nutritional Sciences

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The University of Texas at Austin	B.A.	1971	Microbiology
The University of Texas at Austin	Ph.D.	1985	Biological Sciences

A. Positions and Honors.

Positions and Employment

1971-1973 Research Scientist Assistant, National Institute of Dental Research, Bethesda, Maryland
1973-1986 Research Scientist Assistant/Associate, The University of Texas at Austin
1986-1991 Assistant Professor, The University of Texas at Austin, Nutritional Sciences
1991-1998 Associate Professor, The University of Texas at Austin, Nutritional Sciences
1998-present Professor, The University of Texas at Austin, Nutritional Sciences

Other Experience and Honors

1990-1996 *Journal of Nutritional Immunology*, Member of Editorial Board
1996-2000 National Institutes of Health, Metabolic Pathology Study Section, Member
1996-present American Institute for Cancer Research, Panel II, Member
1999-2000 National Institutes of Health, Member of Special Study Section reviewing "Insight Awards to Stamp Out Breast Cancer" grants
1997, 1998 US Army Medical Research and Materiel Command, Breast Cancer Research Program, Member Peer Review Panel: Clinical and Experimental Therapeutics #4
1997-present *Journal of Nutrition and Cancer*, Associate Editor
2000-present Julian C. Barton Professorship in Nutrition
2002 National Institutes of Health, Member of Special Study Section reviewing "Chemoprevention of ER-negative Breast Cancer" grants
2003 NIH Grant Reviews: NIH, NCI, DEA, SRRB "Molecular Targets for Nutrients in Prostate Cancer Prevention"
2004 NIH Grant Reviews: NIH, NCI, Chemo/Dietary Prevention (CDP) Study Section
2004 NIH Grant Reviews: NIH, NCI, Special Grant Reviews for Cancer Immunology & Immunotherapy and Oncological Sciences Study Sections

B. Selected Peer-Reviewed Publications (in chronological order).

(Total of 71 peer-reviewed publications)

1. Yu, W., K. Israel, Q. Y. Liao, C. M. Aldaz, B. G. Sanders, and K. Kline. 1999. Vitamin E succinate VES) induces Fas sensitivity in human breast cancer cells: Role for Mr 43,000 Fas in VES- triggered apoptosis. *Cancer Research.* 59: 953-961.

2. Yu, W., M. Simmons-Menchaca, A. Gapor, B. G. Sanders, and K. Kline. 1999. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. *Nutrition and Cancer.* 33: 26-32.
3. Herbert, B. S., B. G. Sanders, and K. Kline. 1999. N-(4-hydroxyphenyl) retinamide activation of transforming growth factor-β and induction of apoptosis in human breast cancer cells. *Nutrition and Cancer.* 34:121-132.
4. Israel, K., W. Yu, B. G. Sanders, and K. Kline. 2000. Vitamin E succinate (VES) induces apoptosis in human prostate cancer cells: Role for Fas in VES-triggered apoptosis. *Nutrition and Cancer.* 36:90-100.
5. Kline, K., W. Yu , and B. G. Sanders. 2001. Vitamin E: Mechanisms of action as tumor cell growth inhibitors. *Journal of Nutrition.* 131:161S-163S.
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9. Yu, W., B. G. Sanders, and K. Kline. 2002. RRR-α-tocopheryl succinate-induction of DNA synthesis arrest of human MDA-MB-435 cells involves TGF-β independent activation of p21 (Waf1/Cip1). *Nutrition and Cancer.* 43: 227-236.
10. Lawson, K.A., K. Anderson, M. Menchaca, J. Atkinson, L-Z. Sun, V. Knight, B. E. Gilbert, C. Conti, B. G. Sanders, and K. Kline. 2003. Novel vitamin E analog decreases syngeneic mouse mammary tumor burden and reduces lung metastasis. *Molecular Cancer Therapeutics* 2: 437-444.
11. Yu, W., B. G. Sanders and K. Kline. 2003. RRR-α-tocopheryl succinate-induced apoptosis of human breast cancer cells involves Bax translocation to mitochondria. *Cancer Research* 61: 2483-2491.
12. Kline, K., K. A. Lawson, W. Yu, and B. G. Sanders. 2003. Vitamin E and breast cancer prevention: Current status and future potential. *Journal of Mammary Gland Biology and Neoplasia.* 8: 91-102.
13. Shun, M-C., W. Yu, A. Gapor, R. Parsons, J. Atkinson, B. G. Sanders, and K. Kline. 2004. Pro-apoptotic mechanisms of action of a novel vitamin E analog (α-TEA) and a naturally occurring form of vitamin E (δ-tocotrienol) in MDA-MB-435 human breast cancer cells. *Nutrition and Cancer.* 48: 95-105.
14. Anderson, K., M. Simmons-Menchaca, K. A. Lawson, J. Atkinson, B. G. Sanders and K. Kline. 2004. Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E succinate and vitamin E analogue, α-TEA. *Cancer Research.* 64: 4263-4269.
15. Lawson, K. A., K. Anderson, R. M. Snyder, M. Simmons-Menchaca, J. Atkinson, L-Z. Sun, A. Bandyopadhyay, V. Knight, B. E. Gilbert, B. B. Sanders and K. Kline. 2004. Novel vitamin E analogue and 9-nitro-camptothecin administered as liposome aerosols decrease syngeneic mouse mammary tumor burden and inhibit metastasis. *Cancer Chemotherapy and Pharmacology.* In Press.
16. Zhang, S., K. A. Lawson, M. Simmons-Menchaca, L-Z. Sun, B. G. Sanders, and K. Kline. 2004. Vitamin E analog α-TEA and celecoxib alone and together reduce human MDA-MB-435-FL-GFP breast cancer burden and metastasis in nude mice. *Breast Cancer Research and Treatment.* 87:111-121.
17. Lawson, K. A., K. Anderson, M. Simmons-Menchaca, J. Atkinson, L-Z. Sun, B. G. Sanders, and K. Kline. 2004. Comparison of vitamin E derivatives α-TEA and VES in reduction of mouse mammary tumor burden and metastasis. *Experimental Biology and Medicine.* 229: 954-963.
18. Anderson, K., K. A. Lawson, M. Simmons-Menchaca, L-Z. Sun, B. G. Sanders and K. Kline. 2004. α-TEA plus cisplatin reduces human cisplatin-resistant ovarian cancer cell tumor burden and metastasis. *Experimental Biology and Medicine.* 229: 1169-1176.

C. Research Support.

Ongoing Research Support

Human Breast Cancer Cell Growth Inhibition by Vitamin E
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: DHHS-National Institutes of Health-National Cancer Institute
Award Period: August 1, 2005 - May 31, 2009
The goals of this project are to investigate the ability of alpha-TEA to be an effective chemotherapeutic agent for breast cancer alone and in combination with doxorubicin and to identify and characterized dysregulated signaling pathways/events in human breast cancer cells that are modified by alpha-TEA.
Role: Co-Principal Investigator

Natural and Synthetic Agents for Treatment of Cancer
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: Foundation for Research
Award Period: January 1, 2005 - December 31, 2005
The goals of this project are to identify and characterize analogs of vitamin E that have anti-cancer properties.
Role: Co-Principal Investigator

CORE GRANT
Title: "Mechanisms and Prevention of Environmental Disease"
Director: John DiGiovanni
Funding Agency: DHHS, PHS, NIH, Environmental Health Sciences Core Center Grant
Grant Number: ES07784
Total Award Period: April 1, 2001 through March 31, 2006
There are 26 faculty members from MD Anderson-Science Park Research Division; 13 faculty members from the University of Texas at Austin; and 13 faculty members from MD Anderson-Houston on this large Center Grant.
This grant provides money for the establishment and maintenance of shared service cores:
Core 1: Molecular Biology; Core 2: Transgenic; Core 3: Histology & Tissue Proc.;
Core 4: Flow Cytometry; Core 5: Analytical; and Core 6: Biostatistics.
Members of this core grant receive a discount in fees assessed for the use of these special research services.
Role: Member of Research Core 1 (Mechanisms of Toxicity and Cell Death).

On a competitive basis we were awarded a Pilot Project from the ES07784 core grant.
Title: "α-TEA & Celecoxib in Skin Cancer Prevention"
Participants: Kimberly Kline, Bob G. Sanders and Susan Fischer
Funding Agency: 5 P30 ES07784
Total Award Period: July 1, 2004 through June 30, 2005
The goals of this project are to assess the efficacy of a novel vitamin E analog and celecoxib alone and in combination to prevent UV-B induced skin cancer in the SKH hairless mouse model.
Role: Principal Investigator

TRAINING GRANTS
Title: NIEHS Toxicology Training Grant: "Training in Molecular Toxicology and Environmental Disease"
Director: John Richburg
Participants: Mark Bedford, Claudio Conti, Kevin Dalby, John DiGiovanni, Susan Fischer, Robin Fuchs-Young, David Johnson, James Kehrer, Kimberly Kline, Michael MacLeod, Ellen Richie, Bob Sanders, Dean Tang, Karen Vasquez, and Cheryl Walker
Funding Agency: National Institutes of Health / National Institute for Environmental Health Sciences

Total Award Period: July 1, 2003 through June 30, 2008
This is a training grant for support of eight pre-doctoral trainees. Currently Rachel Snyder, pre-doctoral student in the lab, holds a fellowship awarded by this training grant.
Role: Member

Title: "Short Term Research Training for Minority Students"
Director: John Richburg
Participants: Mark Bedford, Claudio Conti, Kevin Dalby, John DiGiovanni, Susan Fischer, Robin Fuchs-Young, David Johnson, James Kehrer, Kimberly Kline, Michael MacLeod, Ellen Richie, Bob Sanders, Dean Tang, Karen Vasquez, and Cheryl Walker
Funding Agency: National Institutes of Health / National Institute for Environmental Health Sciences
Total Award Period: April, 1, 2002 through March 31, 2007
Role: Member

Pending Research Support

Pilot Project from the ES07784 core grant.
Title: "α-TEA & Selenium in Prostate Cancer Prevention"
Participants: Bob G. Sanders, Claudio Conti and Kimberly Kline
Funding Agency: 5 P30 ES07784
Total Award Period: September 1, 2005 through August 31, 2006
The goals of this project are to assess the efficacy of a novel vitamin E analog and selenium alone and in combination to prevent prostate cancer in the TRAMP mouse model.
Role: Co-Principal Investigator

Completed Research Support

Human Breast Cancer Cell Growth Inhibition by Vitamin E
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: DHHS-National Institutes of Health-National Cancer Institute
Award Period: August 1, 2000 - July 31, 2005
The goals of this project are to identify and characterize the biochemical and cellular events involved in how vitamin E based compounds induced DNA synthesis arrest, cellular differentiation and apoptosis in human breast cancer cells.
Role: Co-Principal Investigator

Natural and Synthetic Agents for Treatment of Cancer
Participants: Kimberly Kline, and Bob G. Sanders
Funding Agency: Foundation for Research
Award Period: January 1, 2004 - December 31, 2004
The goals of this project are to identify and characterize analogs of vitamin E that have anti-cancer properties.
Role: Co-Principal Investigator

Dietary Interactions to Inhibit Mammary Cancer
Participants: Kimberly Kline and Bob G. Sanders
Funding Agency: The State of Texas Coordinating Board ATP
Total Award Period: January 1, 2002 through December 31, 2003 (extended to August 31, 2004)
The goals of this project are to test whether three nutrient-based compounds singly or in combination exhibit enhanced chemotherapeutic and/or chemopreventive properties in different experimental models of breast cancer.
Role: Co-Principal Investigator

Novel Vitamin E Analogue Decreases Syngeneic Mouse Mammary Tumor Burden and Reduces Lung Metastasis[1]

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Abstract

A nonhydrolyzable ether analogue of RRR-α-tocopherol, 2,5,7,8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl)chroman-6-yloxyacetic acid, called RRR-α-tocopheryloxyacetic acid or RRR-α-tocopherol ether-linked acetic acid analogue (α-TEA), exhibits antitumor activity *in vitro* and *in vivo* using a syngeneic BALB/c mouse mammary tumor model (line 66 clone 4 stably transfected with green fluorescent protein). Treatment of cells with 5, 10, and 20 μg/ml α-TEA for 3 days produced 6, 34, and 50% apoptosis, respectively, and treatment of cells with 10 μg/ml for 2, 3, 4, and 5 days produced 20, 35, 47, and 58% apoptosis, respectively. A liposomal formulation of α-TEA administered by aerosol reduced s.c. tumor growth and lung metastasis. α-TEA-treated animals showed a significant decrease in tumor volumes over 17 days of aerosol treatment ($P < 0.001$). Forty percent of aerosol as well as untreated control mice had visible, macroscopic lung metastases *versus* none (0%) of the α-TEA-treated mice. On the basis of fluorescence microscopic examination of the surface (top and bottom) of flattened whole left lung lobes, an average of 60 ± 15 and 102 ± 17 *versus* 11 ± 4 fluorescent microscopic metastases was observed in aerosol control and untreated control *versus* α-TEA-treated animals, respectively. α-TEA formulated in ethanol + peanut oil (5 mg/mouse/day) delivered by gavage did not reduce s.c. primary tumor burden; however, fluorescent microscopic lung metastases were significantly reduced ($P < 0.0021$). In summary, α-TEA formulated in liposomes and delivered by aerosol is a potent antitumor agent and reduces lung metastasis.

Introduction

Several studies have described the potent antitumor activity of RRR-α-tocopheryl succinate (VES),[3] a hydrolyzable ester derivative of RRR-α-tocopherol (natural vitamin E). Prasad and Edwards-Prasad (1) were the first to describe the capacity of VES but not other forms of vitamin E to induce morphological alterations and growth inhibition of mouse melanoma B-16 cells and to suggest that VES might be a useful therapeutic agent for tumors. Additional studies have demonstrated that VES is a potent growth inhibitor of a wide variety of epithelial cancer cell types, including breast, prostate, lung, and colon, as well as hematopoietic-lymphoid leukemia and lymphoma cells, *in vitro* (2–7).

Recent studies have demonstrated VES to have antitumor and antimetastatic activity in animal xenograft and allograft models when administered i.p. (8–12), suggesting a possible therapeutic potential. VES administered i.p. or p.o. has also been shown to have inhibitory effects on carcinogen [benzo(a)pyrene]-induced forestomach carcinogenesis in mice, suggesting potential as an anticarcinogenic agent (13). Investigations have demonstrated that VES induces concentration- and time-dependent inhibition of cancer cell growth via DNA synthesis blockage, induction of cellular differentiation, and induction of apoptosis (5, 6, 10, 14–16).

VES is noteworthy not only for its induction of growth inhibitory effects on tumor cells but also for its lack of toxicity toward normal cells and tissues (2–7, 11). The use of a nonhydrolyzable VES derivative has shown that it is the intact compound and not either of its cleavage products (namely, RRR-α-tocopherol or succinic acid) that are responsible for the antiproliferative effects (4). Thus, the antiproliferative actions of this vitamin E derivative are not related to antioxidant properties.

In an effort to develop a clinically useful vitamin E-based chemotherapeutic agent and to administer it in a clinically

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[2] To whom requests for reprints should be addressed, at Division of Nutrition/A2703, University of Texas at Austin, Austin, TX 78712-1097. Phone: (512) 471-8911; Fax: (512) 232-7040; E-mail: k.kline@mail.utexas.edu.

[3] The abbreviations used are: VES, vitamin E succinate; α-TEA, 2,5,7,8-tetramethyl-2R-(4R, 8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid; 66cl-4-GFP, Balb/c mouse mammary tumor line 66 clone 4 stably transfected with GFP; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase; THF, tetrahydrofuran; VEH, vehicle.

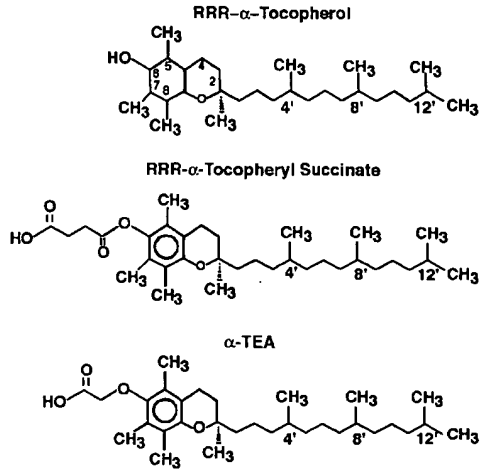

Fig. 1. Structures for RRR-α-tocopherol, VES, and α-TEA. Common names for RRR-α-tocopherol are D-α-tocopherol or natural vitamin E. Chemical name is 2,5,7,8-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol. M_r = 430.69, empirical formula = $C_{29}H_{50}O_2$. Common names for VES are D-α-tocopheryl succinate, D-α-tocopherol acid succinate, and RRR-α-tocopheryl succinate. M_r = 530.76, empirical formula = $C_{33}H_{54}O_5$. Common name for α-TEA is ethyl 6-O-carboxymethyl-α-tocopherol. Chemical name is 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid, referred to as RRR-α-tocopherylo-xyacetic acid or RRR-α-tocopherol ether-linked acetic acid analogue. M_r = 488, empirical formula = $C_{31}H_{52}O_4$.

relevant manner, a nonhydrolyzable ether analogue of RRR-α-tocopherol, namely, α-TEA (Fig. 1) has been produced. As shown in Fig. 1, α-TEA differs from RRR-α-tocopherol by an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage. VES differs from α-TEA in that a succinic acid moiety is linked by an ester linkage to the phenol at carbon 6 of the chroman head (Fig. 1). α-TEA, like VES, is capable of inducing human breast (MCF-7, MDA-MB-231, MDA-MB-435), ovarian (CP-70), cervical (ME-180), endometrial (RL-952), prostate (LnCaP, PC-3, DU-145), colon (HT-29, DLD-1), lung (A-549), and lymphoid (Raji, Ramos, Jurkat) cells to undergo apoptosis. Also like VES, α-TEA does not induce apoptosis in normal human mammary epithelial cells or normal PrEC human prostate epithelial cells.

Because α-TEA is a lipid that is insoluble in water, aerosol delivery of liposomal preparations was chosen as a potentially effective, clinically relevant method of delivery. Aerosol delivery of lipophilic chemotherapeutic agents to mice has been shown to increase drug concentrations in the lungs and other organs compared with i.m. injection, as well as to increase drug effectiveness against breast, lung, and colon xenograft growth in nude mice when compared with either i.m. injection or p.o. administration (17, 18). In addition, this method of delivery appears to be highly effective against pulmonary metastasis of melanoma and osteosarcoma in mice (19). Of major importance, aerosol delivery of drugs shows increased efficacy and is well tolerated by humans (20). α-TEA was tested in this study using either oral (peanut oil VEH) or aerosol (liposome VEH) delivery.

In this article, we report that α-TEA, a novel RRR-α-tocopherol analogue, is a potent concentration- and time-dependent inducer of apoptosis of murine mammary tumor cells *in vitro* and can effectively inhibit s.c. murine mammary tumor burden and lung metastasis when formulated in liposomes and delivered by aerosolization.

Materials and Methods

α-TEA Production in Sufficient Quantity for Animal Studies. For scaled-up production, α-TEA was prepared as follows. NaH (5.0 g, 124.9 mmol) was suspended in dry THF (300 ml) and stirred under argon at 0°C for 10 min before the addition via cabbula of RRR-α-tocopherol (ICN Biomedicals; 41.3 g, 96.1 mmol) that was dissolved in 100 ml of dry THF. This mixture was stirred at 0°C for 15 min while under argon pressure, then ethyl bromoacetate (19.26 g, 115.3 mmol) was added via syringe. The reaction was monitored by thin layer chromatography (hexane:ethyl acetate = 10:1, R_f = 0.65) and was completed in 3.5 h. The reaction mixture was diluted with 150 ml of CH_2Cl_2, washed with saturated NaCl solution (150 ml × 3) until the organic phase was clear, dried over anhydrous Na_2SO_4, and the solvent removed under a reduced pressure. The crude product still contained a small amount of free α-tocopherol, which could be removed by column chromatography on silica gel using hexane:ethyl acetate (30:1 to 20:1) to yield pure product α-TEA ethyl ester (41.6 g, 84%).

The α-TEA ethyl ester (21.0 g, 40.7 mmol) was dissolved in 250 ml of THF, then 75 ml of 10% KOH (122.1 mmol) was added and the mixture stirred at room temperature for 6 h. The reaction was monitored by thin layer chromatography ($CHCl_3$:methanol:CH_3COOH = 97:2.5:0.5, R_f = 0.18) and was quenched with 100 ml of water. The solution was adjusted to pH 3 using 1 N HCl and the product extracted with CH_2Cl_2 (100 ml × 4), washed with saturated NaCl solution, dried over Na_2SO_4, and the solvent was removed under a reduced pressure, providing the final product α-TEA as a white waxy solid (18.5 g, 93%) with a melting point of 54–55°C.

Murine Mammary Tumor Cell Line. 66cl-4-GFP cells are a mouse mammary tumor cell line originally derived from a spontaneous mammary tumor in a Balb/cfC3H mouse and later isolated as a 6-thioguanine-resistant clone (21, 22). Subsequently, these cells were stably transfected with the enhanced GFP by Dr. L-Z. Sun (one of the authors). 66cl-4-GFP cells are highly metastatic with ~40% of animals developing visible macroscopic metastases and 100% of animals developing florescent microscopic metastases in the lungs 26 days after s.c. injection of 2×10^5 tumor cells into the inguinal area. Before use in these studies, cells were sent to the University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia, MO) where they were certified to be pathogen free. 66cl-4-GFP cells were maintained as monolayer cultures in growth media: McCoy's media (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA); 100 μg/ml streptomycin; 100 IU/ml penicillin; 1 × (vol/vol) nonessential amino acids; 1× (vol/vol) MEM vitamins; 1.5 mM sodium pyruvate; and 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO). Treatments were given using this same McCoy's supplemented media, except fetal

bovine serum content was reduced to 5%. Cultures were routinely examined to verify absence of *Mycoplasma* contamination.

Determination of Apoptosis by Morphological Evaluation of DAPI-stained Nuclei. Apoptosis was determined using previously published procedures (23). Briefly, 1×10^5 cells/well in 12-well plates were cultured overnight to permit attachment. Next, the cells were treated with α-TEA, VES (Sigma Chemical Co.), or ethanol control (0.1% ethanol F.C. vol/vol) in experimental media at various concentrations of α-TEA and VES for various time intervals. After treatment, floating cells plus scraped-released adherent cells were pelleted by centrifugation for 5 min at 350 × *g*, washed one time with PBS [137 mM NaCl, 2.7 mM KCL, 10.4 mM Na_2HPO_4, 10.5 mM KH_2PO_4 (pH 7.2)], and stained with 2 μg/ml DAPI (Boehringer Mannheim, Indianapolis, IN) in 100% methanol for 15 min at 37°C. Cells were viewed at ×400 magnification with a Zeiss ICM 405 fluorescent microscope using a 487701 filter. Cells in which the nucleus contained condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Data are reported as percentage of apoptotic cells/cell population (*i.e.,* number apoptotic cells/total number of cells counted). Three different microscopic fields were examined, and 200 cells counted at each location for a minimum of 600 cells counted/slide. Apoptotic data are presented as mean ± SD for three independently conducted experiments.

Western Immunoblot Detection of PARP Cleavage Fragment. PARP cleavage was analyzed as an alternate method for detecting apoptosis. 66cl-4-GFP cells were treated as described above for the DAPI assay. After the PBS wash, cells were suspended in lysis buffer [1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM DTT, 2 mM sodium orthovanadate, 10 μg/ml phenylmethylsulfonyl fluoride] for 30 min at 4°C, vortexed, and supernatants collected by centrifugation at 15,000 × *g* for 10 min. Protein concentrations were determined using the Bio-Rad (Bradford) protein assay (Bio-Rad Laboratories, Hercules, CA), and samples (100 μg/lane) were resolved on 7.5% SDS-polyacrylamide gels electrophoresed under reducing conditions. Proteins were electroblotted onto a nitrocellulose membrane (0.2-μM pore Optitran BA-S-supported nitrocellulose; Schleicher and Schuell, Keene, NH). After transfer, membranes were blocked with blocking buffer [25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 0.5% Tween 20, and 5% nonfat dry milk] for 45 min at room temperature. Immunoblotting was performed using 1 μg of primary rabbit antihuman PARP antibody [PARP (H-250), Santa Cruz Biotechnology, Santa Cruz, CA], and horseradish peroxidase-conjugated goat antirabbit immunoglobulin was used as the secondary antibody (Jackson Immunoresearch Laboratory, West Grove, PA) at a 1:3000 dilution. Horseradish peroxidase-labeled bands from washed blots were detected by enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography (Kodak BioMax film; Rochester, NY).

BALB/c Mice. Female Balb/cJ mice at 6 weeks of age (~25 g in weight) were purchased from Jackson Labs (Bar Harbor, ME) and were allowed to acclimate at least 1 week. Animals were housed at the Animal Resource Center at the University of Texas at Austin at 74 ± 2°F with 30–70% humidity and a 12-h alternating light-dark cycle. Animals were housed 5/cage and given water and standard lab chow (Harlan Teklad no. 2018 Global 18% Protein Rodent Diet; Madison, WI) *ad libitum*. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Tumor Cell Inoculation. 66cl-4-GFP cells were harvested by trypsinization, centrifuged, and resuspended in McCoy's media, containing no supplements, at a density of 2×10^5/100 μl. Mice were injected in the inguinal area at a point equal distance between the fourth and fifth nipples on the right side using a 23-gauge needle.

Fifty mice were assigned (10/group) into five groups [(group 1: untreated control; group 2: liposome/aerosol control; group 3: liposomal α-TEA aerosol treatment; group 4: peanut oil + ethanol/gavage control; group 5: α-TEA in ethanol + peanut oil/gavage treatment) such that the mean tumor volume of each group was closely matched. Each group had an average tumor volume/group = 6.35 mm^3 at the start of treatments, which were begun 9 days after tumor cell inoculation. Ten additional mice, not injected with tumor cells, were treated with aerosol or oral α-TEA (5 each) for 17 days, removed from treatment, and observed for an additional 11 months to evaluate long-term safety. Tumors were measured using calipers every other day, and volumes were calculated using the formula: volume (mm^3) = [width (mm^2) × length (mm)]/2 (24). Body weights were determined weekly.

Preparation of α-TEA Solubilized in Peanut Oil for Delivery by Gavage. α-TEA was dissolved in 100% ethanol (400 mg/ml) and then mixed with peanut oil (100% peanut oil; nSpired Natural Foods, San Leandro, CA) at a ratio of 1:8 (v/v). Control treatment consisted of equivalent amounts of ethanol and peanut oil as contained in the α-TEA treatment. The mixtures were vortexed vigorously, stored at 4°C, and brought back to room temperature and revortexed vigorously immediately before administration.

Preparation of α-TEA Liposomes for Delivery by Aerosol. An α-TEA/liposome ratio of 1:3 (w/w) was determined empirically to be optimal by methods described previously (18). To prepare the α-TEA/lipid combination, the components were first brought to room temperature. The lipid (1,2-dilauroyl-sn-glycero-3-phosphocholine; Avanti Polar-Lipids, Inc., Alabaster, AL) at a concentration of 120 mg/ml was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX) then sonicated to obtain a clear solution. α-TEA at 40 mg/ml was also dissolved in tertiary-butanol and vortexed until all solids were dissolved. The two solutions were then combined in equal amounts (v:v) to achieve the desired ratio of 1:3 α-TEA/liposome, mixed by vortexing, frozen at −80° C for 1–2 h, and lyophilized overnight to a dry powder before storing at −20°C until needed. Each treatment vial contained 75 mg of α-TEA.

Aerosol Delivery. Aerosol was administered to mice as described previously (18). Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 liter/min airflow was used with an AeroTech

II nebulizer (CIS-US, Inc., Bedford, MA) to generate aerosol. The particle size of α-TEA liposome aerosol discharged from the AeroTech II nebulizer was determined using an Anderson Cascade Impactor to have a mass median aerodynamic diameter of 2.01 μm, with a geometric SD of 2.04. About 30% of such particles when inhaled will deposit in the respiratory tract of the mouse and the remaining 70% will be exhaled (18). Aerosolized liposomal drug delivery also includes oral ingestion because of swallowing of nasal and lung mucus secretions, and in mice, there is the added oral ingestion factor created by their inborn grooming behavior, which rapidly facilitates the translocation of any drug deposited onto the fur into the digestive tract (18).

Before nebulization, the α-TEA/lipid powder was brought to room temperature and reconstituted by adding 3.75 ml of distilled water to achieve the final desired concentration of 20 mg/ml α-TEA. The mixture was allowed to swell at room temperature for 30 min with periodic inversion and vortexing and then added to the nebulizer. Mice were placed in plastic cages (7 × 11 × 5 in.) with a sealed top in a safety hood. Aerosol entered the cage via a 1-cm accordion tube at one end and was discharged at the opposite end, using a one-way pressure release valve. Animals were exposed to aerosol until all α-TEA/liposome was aerosolized (~15 min).

Oral Delivery. α-TEA/peanut oil mixture was brought to room temperature and revortexed vigorously immediately before administration by gavage 100 μl/mouse/day (final concentration 5 mg α-TEA/mouse/day).

Lung Metastasis. Macroscopic metastases in all five lung lobes were counted visually at time of sacrifice. Fluorescent microscopic metastases were counted using a Nikon fluorescence microscope (TE-200; ×200 magnification). For analyses, lung tissue (left lung lobes) was flattened, and the entire surface (top and bottom) scored for fluorescent green microscopic metastases. Fluorescent microscopic metastases were scored by size into three size grouping: <20 μm, 20–50 μm, and >50 μm. On the basis of a typical 66cl-4-GFP tumor cell size of 10–20 μm in diameter, the <20-μm grouping is thought to represent solitary cells; the 20–50-μm grouping two to five cells; and the >50-μm grouping microscopic metastases of greater than two to five cells.

Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling Assay for Detection of Apoptosis *in Vivo*. Deparaffinized sections (5 μm) of tumor tissue were used to assess apoptosis using reagents supplied in the ApopTag *in Situ* Apoptosis Detection kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Nuclei that stained brown were scored as positive for apoptosis and those that stained blue were scored as negative. At least 16 microscopic fields (×400) were scored/tumor. Data are presented as the mean ± SE number of apoptotic cells counted in three separate tumors from each group.

Statistical Analyses. Animal numbers for experiments were determined by power calculations derived from data generated by preliminary pilot studies. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor ANOVA using SPSS (SPSS, Inc., Chicago, IL). Difference in number of fluorescent microscopic metastases/group were determined using the Mann-Whitney rank test using Prism software version 3.0 (Graphpad, San Diego, CA). A level of $P < 0.05$ was regarded as statistically significant.

Results

VES and α-TEA Induce Apoptosis in 66cl-4-GFP Cells, *in Vitro*. Previous studies indicate that VES is a potent apoptotic inducer in many human cancer cell lines, including breast cancer. For comparative purposes, we included VES in the *in vitro* analyses of α-TEA induced apoptosis. BALB/c mammary cancer 66cl-4-GFP cells were treated with VES or α-TEA, and apoptosis was assessed by morphological analyses of DAPI-stained cells for condensed nuclei and fragmented DNA.

Nuclei from 66cl-4-GFP cells treated with 10 μg/ml α-TEA or VES for 3 days exhibited condensed and fragmented DNA, characteristics of apoptosis, whereas nuclei from untreated cells did not exhibit these morphological characteristics (Fig. 2*A*). The level of apoptosis of 66cl-4-GFP cells treated for 3 days with 2.5, 5, 10, and 20 μg/ml α-TEA or VES in comparison to controls was 2.5-, 3-, 17- and 24-fold higher for α-TEA and 1.5-, 2.5-, 8-, and 17-fold higher for VES (Fig. 2*B*). Untreated, VEH, and ethanol controls exhibited background levels of apoptosis: 2, 2, and 3%, respectively (Fig. 2*B*).

α-TEA was shown to induce apoptosis in a time-dependent manner. 66cl-4-GFP cells treated with 10 μg/ml α-TEA for 2–5 days exhibited 10-, 18–24-, and 28-fold increases in apoptosis over baseline (2%), respectively (Fig. 2*C*). Induction of apoptosis was confirmed by the presence of PARP cleavage after treatment of 66cl-4-GFP cells with 5, 10, and 20 μg/ml α-TEA for 48 h (Fig. 2*D*). The M_r 84,000 cleavage fragment of PARP was evident at both 10 and 20 μg/ml α-TEA treatment, whereas only intact PARP protein was detected in cells treated with 5 μg/ml α-TEA or in the untreated control cells (Fig. 2*D*).

Aerosol Characteristics of α-TEA Incorporated into Liposomes. High-performance liquid chromatography analyses were conducted on α-TEA liposomes recovered from aerosol collected with an All Glass Impinger (Ace Glass Co., Vineland, NJ). An estimate of the amount of aerosolized α-TEA delivered/mouse/treatment was derived from the following formula (18): delivered drug dose = drug concentration (μg/liter) × volume of air intake/min/unit of body weight (1 ml/min/g body weight) × duration of drug delivery in minutes × estimated percentage of aerosolized drug deposited in the respiratory tract, which includes the nose, trachea, and lungs (30%). On the basis of this formula, we estimate that 36 μg of α-TEA were deposited in the respiratory tract of each mouse each day. Thus, for the 17-day treatment period, we estimate that each mouse received 612 μg of α-TEA from liposomal aerosol delivery. Although mice received 5 mg/day for 13 days of treatment by gavage for a total of 65 mg, we do not know the bioavailability of α-TEA delivered by this method.

Liposomal α-TEA/Aerosol Treatment Suppressed 66cl-4-GFP Tumor Growth in BALB/c Mice and Reduced Lung Macroscopic and Microscopic Metastases. Mean tumor volumes of the liposomal α-TEA/aerosol treatment group, in comparison to aerosol control, was significantly lower over

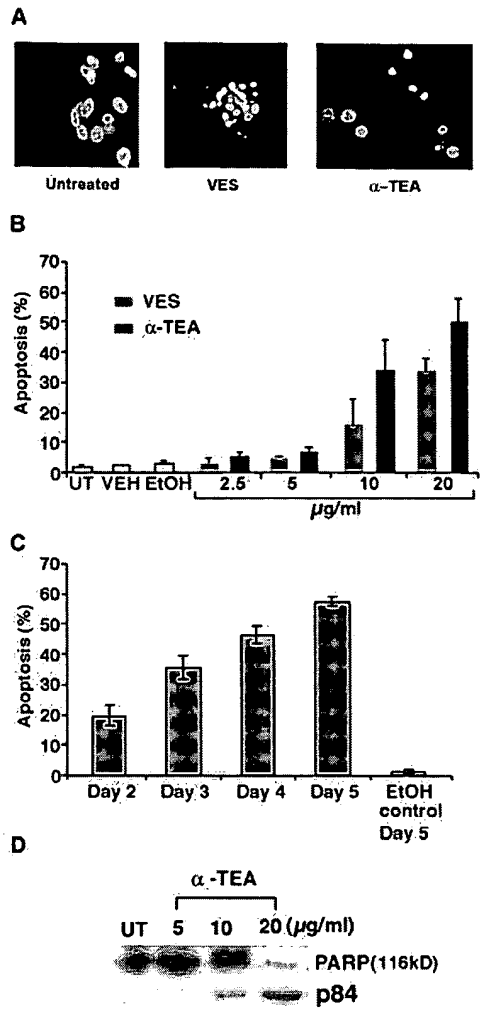

Fig. 2. Documentation of α-TEA-induced apoptosis. *A*, 66cl-4-GFP murine mammary cells were treated with 10 μg/ml α-TEA or VES (positive control) or untreated and cultured for 3 days. Cells were harvested, nuclei were labeled with the fluorescent DNA binding dye DAPI, and cells were examined using a Zeiss ICM 405 fluorescent microscope (×400), using a 487701 filter. Nuclei of cells with condensed chromatin or fragmented nuclei were scored as apoptotic. Data are representative of numerous experiments. *B* and *C*, analyses of nuclei of DAPI-stained cells show α-TEA to induce apoptosis in a concentration- and time-dependent manner. Data are depicted as mean ± SD of three separate experiments. *D*, additional evidence of α-TEA induction of apoptosis by PARP cleavage. 66cl-4-GFP cells were treated with 5, 10, or 20 μg/ml α-TEA for 48 h, and cellular lysates were analyzed for PARP cleavage by western immunoblot analyses. Data are representative of three separate experiments.

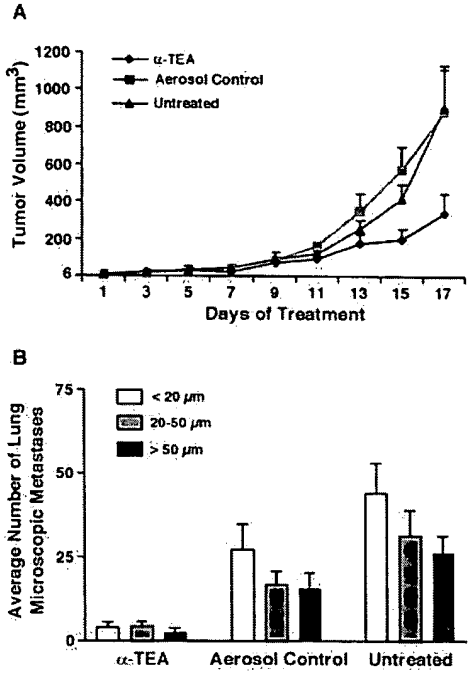

Fig. 3. Liposomal α-TEA delivery by aerosol inhibits tumor burden and microscopic metastases in the lung. *A*, 66cl-4-GFP cells at 2×10^5/mouse were injected into the inguinal area at a point equal distance between the fourth and fifth nipples. Nine days after tumor injection, mice were assigned to control and α-TEA treatment groups such that the mean tumor volume of each group was closely matched (average tumor volume/group = 6.35 mm^3). Mice (10/group) were not treated or treated daily with liposomal α-TEA/aerosol or aerosol only for 17 days. Tumor volume/mouse was determined at 2-day intervals. Tumor volumes (mm^3) are depicted as mean ± SE. *B*, with the aid of a Nikon fluorescent microscope, the number of fluorescent microscopic metastases on the surface (top and bottom) of flattened left lung lobes from liposomal α-TEA/aerosol (8 mice), aerosol only (10 mice), and untreated mice (10 mice) were determined. Data are depicted as mean ± SE.

Table 1 66cl-4-GFP mammary cancer cell lung metastasis in Balb/c mice receiving liposomal α-TEA or liposome control by aerosol or no treatment

Treatments	No. of animals/group with visible lung macroscopic metastases[a]	No. of visible lung macroscopic metastases/animal[b]
No Treatment	4/10	3.25 ± 1.7
Aerosol/liposome control	4/10	4.25 ± 0.5
Aerosol/liposomal α-TEA	0/10	0

[a] Macroscopic metastases in all five lung lobes for each animal in all treatment groups were counted visually at the time of sacrifice.
[b] Data are expressed as the mean ± SD of visible lung macroscopic metastases observed in the four lung macroscopic metastases bearing animals in the two control groups.

17 days of treatment ($P < 0.001$; Fig. 3*A*). At sacrifice, all five lung lobes from each animal were examined visually for macroscopic metastases. No visible macroscopic metastases were seen in the α-TEA treatment group, whereas 40% each of untreated and aerosol control animals exhibited macroscopic metastases with an average of 3.25 ± 1.7 and 4.25 ± 0.5 visible tumors/animal, respectively (Table 1). Use of a Nikon fluorescence microscope permitted measurement of green fluorescing microscopic metastases into three size groupings (<20 μm, 20–50 μm, and >50 μm). Because the tumor cells are ~10–20 μm in diameter, the microscopic metastases scored as <20 μm most likely represent single cells. This analysis showed a decrease in microscopic metastases of all three size groupings in the α-TEA treatment group in comparison to either the aerosol or untreated controls (Fig. 3*B*). The mean number of microscopic metastases in the α-TEA treatment group (11.4 ± 3.5; $n = 8$), in com-

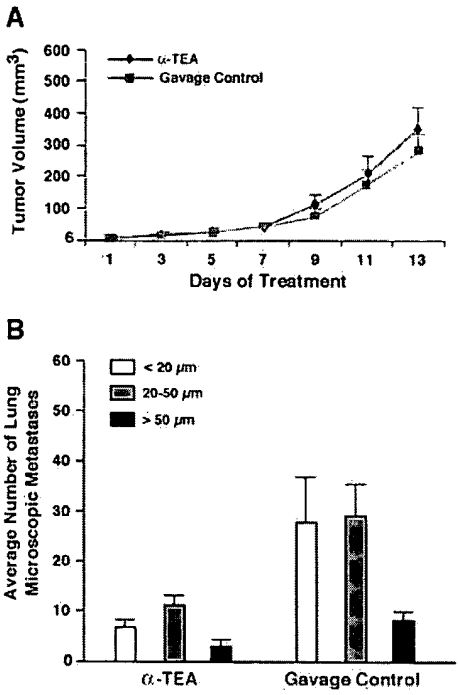

Fig. 4. *A* and *B*, α-TEA administered by gavage does not inhibit primary tumor burden but does reduce lung microscopic metastases. These studies were conducted in parallel with the studies described in Fig. 3 and differ only in that the mice were treated daily by gavage with 5 mg of α-TEA dissolved in ethanol and peanut oil or ethanol + peanut oil VEH control and were treated for only 13 days. Tumor volume and lung microscopic metastases data were determined as described in the legend to Fig. 3. Data are depicted as mean ± SE.

parison to aerosol control (60.0 ± 15; $n = 10$), was significantly reduced ($P < 0.002$). Although the mean number of microscopic metastases in the aerosol control group *versus* the untreated control ($n = 10$) was reduced (60 ± 15.2 *versus* 101.7 ± 17.0), the difference was not significant ($P < 0.063$; Fig. 3*B*).

Although this exact experiment has not been repeated, we have conducted several studies evaluating the antitumor properties of α-TEA using the 66cl-4-GFP syngeneic mammary cancer model. Data from experiments comparing the antitumor properties of aerosolized α-TEA formulated in liposomes with different aerosolized vitamin E compounds, and data from an experiment comparing α-TEA alone and in combination with 9-nitrocamptothecin, consistently and repeatedly show that tumor volume, visible macroscopic lung metastases, and fluorescent microscopic lung metastases are significantly reduced in comparison to controls (unpublished data).

Delivery of α-TEA by Gavage Did Not Reduce Tumor Burden at the s.c. Inoculation Site but Did Reduce the Number of Lung Microscopic Metastases. In contrast to liposomal α-TEA/aerosol treatment, mean tumor volumes from mice receiving 5 mg/day/mouse α-TEA formulated in peanut oil by gavage did not differ from the mean tumor volume of the gavage control (Fig. 4*A*). However, administration of α-TEA by gavage significantly reduced the number of lung microscopic metastases (21.2 ± 3.5 *versus* 65.5 ± 15.3; $P < 0.0021$; $n = 10$ for both groups). The average numbers of microscopic metastases, based on three size groupings (<20 μm, 20–50 μm, >50 μm), were 6.8 ± 1.5, 11.3 ± 1.8, and 3.1 ± 1.2 for mice administered α-TEA by gavage, whereas average numbers of microscopic metastases in gavage control mice were 27.9 ± 9.0, 29.2 ± 6.3, and 8.4 ± 1.5, respectively (Fig. 4*B*).

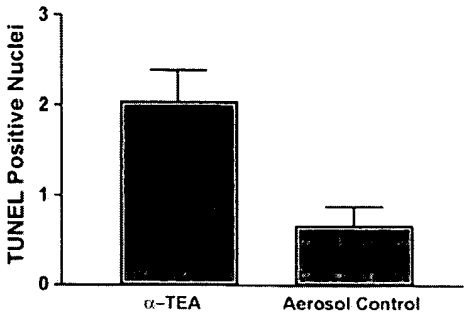

Fig. 5. α-TEA induces 66cl-4-GFP cells to undergo apoptosis *in vivo*. α-TEA induction of apoptosis was determined using 5-μm tumor sections derived from liposomal α-TEA/aerosol treatment and liposome aerosol control group animals ($n = 3$). Apoptotic cells were determined using ApopTag *in Situ* Apoptosis Detection kit (Intergen, Purchase, NY).

No differences in mean body weights among any of the treatment or control groups were observed (data not shown). Nontumor-bearing mice that were treated with either aerosol/α-TEA or gavage/α-TEA for 17 days and then kept for 11 months to assess long-term effects did not show any adverse effects of the α-TEA treatments.

Histological Evaluation of Tumors. Tumors from aerosol control and α-TEA-treated animals were examined after H&E staining. No evidence for α-TEA-induced differentiation was seen because tumors from both control and α-TEA-treated animals were judged to be poorly differentiated spindle cell carcinomas with high mitotic index.

Induction of Apoptosis by α-TEA *in Vivo*. In view of the *in vitro* data showing that α-TEA inhibits 66cl-4-GFP tumor cell growth via induction of apoptosis, three tumors from each of the liposomal α-TEA/aerosol treatment and aerosol control groups were examined for apoptosis using terminal deoxynucleotidyl transferase-mediated nick end labeling staining of 5-μm tumor sections. Tumors from mice treated with α-TEA had a mean ± SE of 2.04 ± 0.23 apoptotic cells/field, whereas tumors from aerosol control mice had a mean ± SE of 0.67 ± 0.15 apoptotic cells/field (Fig. 5).

Discussion

Analyses of natural occurring vitamin E compounds and vitamin E analogues for antitumor activity for breast cancer has been reviewed recently (25). Our goal in developing α-TEA is to produce and characterize a tocopherol-based antitumor agent with favorable characteristics suitable for use in humans. Studies reported here demonstrate that the novel vitamin E derivative referred to as α-TEA is an effective antitumor agent, inducing 66cl-4-GFP cells to undergo ap-

optosis both *in vitro* and *in vivo*. Furthermore, α-TEA reduces lung metastasis and does not exhibit toxicity to normal cells and tissues *in vivo*.

Structurally, vitamin E (RRR-α-tocopherol) consists of a chroman head with two rings: one phenolic and one heterocyclic and a saturated phytyl tail (Fig. 1; Ref. 26). α-TEA is a synthetic derivative of vitamin E (RRR-α-tocopherol) with a nonhydrolyzable acetic acid moiety attached to the no. 6 carbon of the phenolic ring of the chroman head by an ether linkage (Fig. 1). In contrast, VES has an ester-linked succinic acid moiety attached to the no. 6 carbon of the phenolic ring of the chroman head (Fig. 1). Thus, α-TEA should be resistant to hydrolysis by cellular esterases, possibly providing a superior apoptotic inducing agent for *in vivo* use in comparison to VES.

This is the first article of the antitumor properties of α-TEA, both *in vitro* and *in vivo*. There are numerous studies showing that natural vitamin E (RRR-α-tocopherol) does not possess antitumor properties for epithelial cells and that VES can induce tumor cells of epithelial origin to undergo apoptosis (1–13, 16). VES has been shown to exhibit antitumor properties when administered i.p. but not by gavage, with the exception of experiments involving stomach cancer (8–13). In this study, we choose to administer α-TEA either via liposomal/aerosol or gavage, two established delivery methods for treatment of cancers both in clinical and home environments.

Administration of α-TEA by aerosol was superior to administration by gavage in these studies in that α-TEA administered by gavage did not reduce tumor burden at the site of s.c. tumor injection in comparison to tumor burden of control mice. Nevertheless, it is of interest that the number of lung microscopic metastases were reduced in comparison to control when α-TEA was administered by gavage, suggesting that α-TEA might be effective via this route of administration. Recent preliminary experiments used gavage delivery of a liposomal α-TEA formulation rather than the ethanol-peanut oil α-TEA formulation used in these studies, and treatments were administered twice a day to achieve a higher daily dose (6 mg/day/mouse) rather than the once a day treatment schedule used in these studies to achieve a total daily dose of 5 mg/day/mouse. Liposomal α-TEA delivered by gavage significantly reduced tumor burden ($P < 0.001$) and resulted in zero animals exhibiting lung macroscopic metastases and significantly reduced lung microscopic metastases in comparison to gavage control (21.5 ± 4.9 *versus* 52.7 ± 4.2; $P < 0.0005$) after 21 days of treatment (unpublished data).

Regarding lung metastasis, it is important to point out some differences in the experimental protocol used in these studies in comparison with studies describing the metastatic nature of the parental 66cl-4 cells (22). In the studies by Miller *et al.* (22), the parental 66cl-4 cells were injected s.c., permitted to grow to 12 × 12-mm (4–7 weeks after tumor cell injection), primary tumors were surgically removed, and animals were sacrificed 3 weeks later. Using this experimental protocol, 89% of the animals exhibited macroscopic metastases. In contrast, in our studies, the GFP-transfected subline of 66cl-4 cells were injected s.c., the primary tumors were permitted to grow for 26 days at which time both primary tumors and lung metastases, both visible macroscopic and fluorescent microscopic metastases, were assessed. Using this experimental protocol, 40% of control animals (both untreated control and aerosol control) had visible macroscopic metastases in the lungs, whereas none (0%) of the α-TEA-treated animals had visible macroscopic metastases in the lungs (Table 1), whereas 100% of control and α-TEA-treated animals exhibited fluorescent microscopic metastases.

Regarding the <20-μm grouping of microscopic metastases, based on recent studies by Chambers *et al.* (27, 28), solitary tumor cells such as these may be potential contributors to dormancy, and if such cells remain viable in sufficiently large numbers, they could contribute to metastatic recurrence after a period of clinical dormancy. Whether the fluorescent microscopic metastases seen in the studies reported here represent viable cells, namely cells that could be removed and demonstrated to grow both in cell culture and after injection into the mammary fat pad, remains to be determined. Nevertheless, it is interesting to note that α-TEA was very effective in markedly decreasing the number of these small fluorescent microscopic metastases, as well as the larger microscopic metastases after both aerosol or oral treatment.

In an effort to try to address the question of whether or not α-TEA is preventing tumor cells from trafficking from the primary s.c. tumor to the lungs via the lymphatic system, we have counted fluorescent tumor cell foci in the axillary and brachial lymph nodes. In these experiments we observed that aerosolized, liposomal formulated α-TEA treatments significantly reduce the number of fluorescent tumor cell foci in the lymph nodes in comparison to aerosol treated controls (0.38 ± 0.1 *versus* 7.0 ± 1.6; $P < 0.0001$). This suggests that α-TEA may be having an effect on the process of metastasis, but more studies are needed (unpublished data).

In summary, data reported here are promising in that they show that a novel vitamin E analogue exhibits the ability to decrease primary tumor burden and reduce lung metastasis in a rather rapid and aggressive syngeneic tumor model without any overt toxic effects when administered by a clinically relevant route, namely, aerosol delivery. Increased rates of tumor cell apoptosis imply that the antitumor effect is due, at least in part, to analogue triggering of tumor cell death. The mechanism of how α-TEA reduces lung metastasis in this model system is unknown and warrants additional investigation.

Acknowledgments

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Comparison of Vitamin E Derivatives α-TEA and VES in Reduction of Mouse Mammary Tumor Burden and Metastasis

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A novel nonhydrolyzable ether derivative of RRR-α-tocopherol, RRR-α-tocopherol ether acetic acid analog [2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA)], and a hydrolyzable ester derivative RRR-α-tocopheryl succinate (vitamin E succinate; VES) inhibited BALB/c mouse 66cl-4-GFP mammary tumor cell growth *in vitro* and *in vivo*. Treatment of 66cl-4-GFP cells in culture with α-TEA or VES induced dose-dependent DNA synthesis arrest and apoptosis and inhibited colony formation. Liposomal formulations of α-TEA delivered orally or by aerosol significantly reduced subcutaneous 66cl-4-GFP tumor burden and metastasis to lung and lymph nodes. Liposomal formulations of VES delivered by aerosol significantly reduced tumor burden and lung metastasis, but not lymph node metastasis. Unlike α-TEA, VES was ineffective in reducing tumor burden and metastasis to lungs and lymph nodes when administered orally. Analyses of tumor sections showed that α-TEA delivered by either method significantly reduced tumor cell proliferation as measured by Ki67, and increased apoptosis as measured by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL), whereas VES delivered by aerosol reduced tumor cell proliferation and increased apoptosis, but not significantly. In summary, the nonhydrolyzable ether vitamin E derivative α-TEA was effective in reducing tumor burden and metastasis when delivered either by aerosol or orally, whereas the hydrolyzable ester vitamin E derivative VES was effective only when delivered by aerosol. Exp Biol Med 229:954–963, 2004

Key Words: vitamin E analog α-TEA; RRR-α-tocopheryl succinate (VES); metastasis; antitumor agents; syngeneic mouse mammary cancer model

Introduction

Our laboratory has been investigating the antitumor properties of natural and synthetic vitamin E compounds, with major emphasis on using human epithelial breast cancer cells in culture to analyze the cellular, molecular, and biochemical events involved in the ability of a succinate ester derivative of vitamin E (RRR-α-tocopherol), RRR-α-tocopheryl succinate (vitamin E succinate; VES) to induce DNA synthesis arrest, differentiation, and apoptosis (1–10). Vitamin E succinate has been shown by this laboratory and others to be a potent inhibitor of epithelial cancer cell growth, inducing human breast, prostate, lung, colon, cervical, and endometrial cancer cells to undergo apoptosis in culture, but not normal human mammary epithelial cells or normal prostate epithelial cells (1–3, 7–9).

Although VES has proven to be a potent anticancer agent *in vitro* and has provided important insights into anticancer signaling pathways, its basic structure has the potential of compromising its potency *in vivo*; namely, the ester linkage can be hydrolyzed by cellular esterases, yielding vitamin E (RRR-α-tocopherol) and succinic acid, neither of which exhibit anticancer properties (1, 9).

In an effort to overcome the potential problem of cellular esterases hydrolyzing the ester-linked succinate moiety of VES and rendering VES ineffective as an anticancer agent, our laboratory has developed a non-hydrolyzable ether analog of RRR-α-tocopherol, namely, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chro-

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man-6-yloxyacetic acid (RRR-α-tocopheryloxyacetic acid or RRR-α-tocopherol ether-linked acetic acid analog [α-TEA]), that exhibits similar anticancer properties to VES in cell culture (11).

Like VES, the parent compound for making α-TEA is natural vitamin E (RRR-α-tocopherol). α-TEA differs from VES in that it has an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage, whereas VES has a succinic acid moiety linked by an ester linkage at this site (12). α-TEA, like VES, is a potent anticancer agent and does not induce apoptosis in normal human mammary epithelial cells (11–13). Expectations were that the ether derivative would be more stable and a better *in vivo* anticancer agent.

BALB/c mammary tumor cell line 66cl-4-GFP, which was originally derived from a spontaneously arising mammary adenocarcinoma and subsequently was stably transfected with the enhanced green fluorescent protein (EGFP), was used in these studies because this cell line, when transplanted into BALB/c mice, exhibits an aggressive tumor with metastasis to lungs and lymph nodes (12). Liposomal formulation of each compound was chosen for this study because α-TEA and VES are lipids and insoluble in water, and α-TEA/peanut oil formulation delivered orally was ineffective (12). Liposome formulations of α-TEA and VES can be administered several ways, including aerosol or gavage. Delivery of lipophilic chemotherapeutic agents to mice by aerosol increased drug concentrations in the lungs and other organs compared with intramuscular or oral administration, and this method of drug delivery has been shown to be highly effective against pulmonary metastasis of melanoma and osteosarcoma in mice (14–16).

We compared the anticancer properties of α-TEA and VES *in vitro* and *in vivo*. *In vitro*, both vitamin E derivatives were effective anticancer agents, inducing DNA synthesis arrest and apoptosis as well as inhibiting colony formation. *In vivo*, α-TEA was superior to VES, significantly reducing tumor burden and metastasis regardless of method of delivery.

Materials and Methods

VES and α-TEA. Vitamin E succinate (formula weight = 530.8) was purchased from Sigma Chemical Co. (St. Louis, MO). Synthesis of α-TEA (formula weight = 488.8) was performed by one of the authors (J.A) and has been described in detail (12).

66cl-4-GFP Murine Mammary Tumor Cell Line. 66cl-4 cells were derived from a spontaneous mammary tumor in a BALB/cfC3H mouse and isolated as a 6-thioguanine-resistant clone (17, 18). These cells were stably transfected with an expression vector of the enhanced green fluorescence protein (EGFP: pEGFP-N1 from BD Biosciences Clontech Laboratories, Inc. Palo Alto, CA) and transfected cells sorted with fluorescent-activated cell sorter (FACS) twice to obtain a population of brightly fluorescing cells. 66cl-4-GFP cells have been shown to be highly metastatic, with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing microscopic metastases detectable with fluorescent microscopy in the lungs 26 days following subcutaneous injection of 2×10^5 tumor cells into the inguinal area (12). 66cl-4-GFP cells were maintained as monolayer cultures, and *in vitro* experiments were performed as previously described (12).

Determination of DNA Synthesis by Incorporation of Tritiated Thymidine. Effects of α-TEA and VES on inhibition of DNA synthesis of 66cl-4-GFP cells were determined by tritiated thymidine incorporation as described previously (6, 19). Briefly, 66cl-4-GFP cells at 2.0×10^4 cells in 0.2 ml volume/well in 96 well plates were cultured with 2.5, 5, 10, or 20 μg/ml of α-TEA (2.6, 5.1, 10.2, 20.5 μ*M*, respectively) or VES (2.3, 4.7, 9.4, 18.8 μ*M*, respectively) for 24 hrs. During the last 6 hrs of incubation, cultures were pulsed with 0.5 μCi tritiated thymidine/well, cells were harvested, and tritiated thymidine uptake was measured using a Beckman LS5000TD liquid scintillation counter (Beckman Coulter, Fullerton, CA). Percent DNA synthesis arrest was determined by comparing the tritiated thymidine uptake (cpm) of treatment groups to tritiated thymidine uptake (cpm) of untreated controls.

Determination of Apoptosis by Morphological Evaluation of 4′,6-Diamidino-2-Phenylindole (DAPI)-Stained Nuclei. Apoptosis was determined using previously published procedures and criteria (2, 12). Data are reported as percentage of apoptotic cells per cell population (i.e., number apoptotic cells/total number of cells counted). For each sample, three different microscopic fields were examined and 200 cells counted at each location for a minimum of 600 cells counted per slide. Apoptotic data are presented as mean ± SD for three independent experiments.

Colony Forming Assay. Effects of α-TEA and VES on colony formation of 66cl-4-GFP cells were determined as previously described (20). Briefly, 66cl-4-GFP cells were seeded in 35 × 10 mm tissue culture plates (Nunclon, Rochester, NY) at increasing cell numbers ranging from 5×10^2 to 1×10^5 cells/plate. Cells were allowed to adhere overnight, then treated with 1.25, 2.5, 5, or 10 μg/ml α-TEA or VES or untreated for 10 days. After 10 days, media were removed; cells were washed in phosphate-buffered saline (PBS) and stained with 0.1% methylene blue in PBS. Plating efficiency was determined by dividing the number of colonies present after 10 days in the untreated plate by the number of cells seeded. The surviving fraction of treated samples was determined as number of colonies present divided by number of cells seeded × plating efficiency.

BALB/c Mice. Female BALB/cJ mice at 6 weeks of age (~25 g body weight) were purchased from Jackson Labs (Bar Harbor, ME) and allowed to acclimate for 1 week. Mice were housed, 5/cage, given water and standard lab chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison, WI) *ad libitum* at the Animal Resource

Center at the University of Texas at Austin and maintained in an environment of 74 ± 2°F with 30%–70% humidity and a 12-hour alternating light-dark cycle. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Tumor Cell Inoculation. 66cl-4-GFP cells were harvested by trypsinization, collected by centrifugation, and resuspended at a density of 2×10^5 cells/100 μl in McCoy's media, containing no supplements. Mice were injected with 2×10^5 cells/100 μl in the inguinal area at a point equal distance between the fourth and fifth nipples on the right side using a 25-gauge needle.

Mice, 10/group, were placed in 6 groups (liposome/aerosol control, liposome/gavage control, liposomal α-TEA/aerosol, liposomal VES/aerosol, liposomal α-TEA/gavage, and liposomal VES/gavage) so that the average tumor volume for all groups was closely matched. Each group had an average tumor volume/group of 0.569 mm^3, 0.450 mm^3, 0.519 mm^3, 0.375 mm^3, 0.613 mm^3, and 0.681 mm^3, respectively, at the start of treatments, which were begun 9 days following tumor cell inoculation. Tumors were measured using calipers every other day, and volumes were calculated using the following formula: volume (mm^3) = [width (mm^2) × length (mm)]/2 (21). Body weights were determined weekly.

Preparation of Liposomal α-TEA and VES. An α-TEA or VES/liposome ratio of 1:3 (w/w) was determined empirically to be optimal by methods previously described (12, 14). To prepare the α-TEA or VES/lipid combinations, the components were first brought to room temperature. The lipid [1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC); Avanti Polar-Lipids, Inc., Alabaster, AL], at a concentration of 120 mg/ml, was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX) and then sonicated to obtain a clear solution. α-TEA or VES at 40 mg/ml was dissolved in tertiary-butanol and vortexed until all solids were dissolved. The lipid DLPC and α-TEA or DLPC and VES were then combined in equal amounts (v:v) to achieve the desired ratio of 1:3 α-TEA or VES/liposome, mixed by vortexing, frozen at –80°C for 1–2 hrs, and lyophilized overnight to a dry powder prior to storing at –20°C until needed.

Aerosol and Gavage Delivery. Aerosol was administered to mice as previously described (12, 14). Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 L/min airflow was used with an AeroTech II nebulizer (CIS-US, Inc., Bedford, MA) to generate aerosol. Particle size and stability of α-TEA and VES liposomes after discharge from the AeroTech II nebulizer was determined using an Anderson Cascade Impactor. The mass median aerodynamic diameter was approximately 2 μm for both vitamin E compounds. Also, it was determined that the formulations were stable; namely, no chemical/physical alterations occurred throughout the 15-min nebulization process. (Note: Once resuspended in water, the liposomal formulations appear to be very stable for hours before or after aerosolization.)

Mice were placed in plastic cages (7 × 11 × 5 in.) with a sealed top in a safety hood. Aerosol entered the cage via a 1-cm accordion tube at one end and was discharged at the opposite end, using a one-way pressure release valve. Mice were exposed to aerosol until all liposomal α-TEA, liposomal VES, or liposome only (control) was aerosolized (~15 mins). Aerosol treatments were conducted once a day, 7 days per week, for a total of 21 days. High-performance liquid chromatography analyses were conducted on α-TEA liposomes recovered from aerosol collected with an All Glass Impinger (Ace Glass Co., Vineland, NJ). An estimate of the amount of aerosolized α-TEA delivered/mouse/treatment was derived from the following formula (12): delivered drug dose = drug concentration (μg/liter) × duration of drug delivery in minutes × estimated percentage of aerosolized drug deposited in the respiratory tract, which includes the nose, trachea, and lungs (30%). On the basis of this formula, we estimate that 36 μg of α-TEA or VES were deposited in the respiratory tract of each mouse each day. Thus, for the 21-day treatment period, we estimate that each mouse received 756 μg of α-TEA or VES from liposomal aerosol delivery (12).

For gavage treatments, lyophilized preparations of liposomal α-TEA, liposomal VES, or liposome only were brought to room temperature and reconstituted by adding 2.5 ml distilled water to achieve the final desired concentration of 32 mg/ml α-TEA or VES. (Note: As mentioned above, once resuspended in water, the liposomal formulations are stable for several hours.) Treatments were vortexed vigorously immediately prior to administration by gavage, 100 μl/mouse at two different times each day, ~8 hours apart. Particle size range of α-TEA and VES liposomes delivered orally was determined to be 4–10 μm. The total amount of α-TEA, VES, or liposome control administered by gavage was 200 μl/mouse per day (final concentration, 6.4 mg α-TEA or VES/mouse/day). Gavage treatments were given twice per day, 7 days/week, for a total of 21 days. Thus, for the 21-day treatment period, each mouse received 134 mg of α-TEA or VES via gavage delivery; however, we do not know the bioavailability of α-TEA or VES delivered by this method.

Lung and Lymph Node Metastasis. Macroscopic metastases in all five lung lobes were counted visually at time of sacrifice (21 days after treatment initiation). Fluorescent microscopic lung metastases were counted as described previously, using a Nikon fluorescence microscope (TE-200; ×200 magnification; Ref. 12). For analyses of lung tissue, left lung lobes were flattened and the entire surface (top and bottom) scored for fluorescent green microscopic metastases. For analyses of axillary and brachial lymph nodes, the tissues were flattened and scored for fluorescent green microscopic metastases. Fluorescent microscopic metastases were scored by size into three size groupings: <20 μm, 20–50 μm, and >50 μm. On the basis

of a typical 66cl-4-GFP tumor cell size of 10–20 μm in diameter, the <20-μm grouping is thought to represent solitary cells; the 20–50-μm grouping two to five cells; and the >50-μm grouping microscopic metastases of greater than two to five cells.

Ki-67 Staining for Detection of Proliferation *In Vivo*. Tumors were collected at the time of sacrifice, 21 days post–treatment initiation. Deparaffanized sections (5-μm) of tumor tissue were used to assess proliferation using antibody to the Ki-67 antigen that is a nuclear antigen expressed in proliferating cells and serves as an indicator of the number of cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked using a 3% H_2O_2 solution for 10 mins, followed by washing with PBS. Rabbit serum (10% v/v in PBS) was applied to sections in order to block nonspecific antibody binding. Sections were incubated with primary Ki-67 antibody (rat-anti-mouse Ki-67 antibody; DAKO Corp., Carpinteria, CA; 1:200 dilution) overnight at 4°C. After primary antibody incubation, slides were incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 mins at room temperature. Tissue sections were then incubated with avidin-biotin complex (ABC-HRP; Vector Laboratories) for 30 mins at room temperature. Immunoreactivity was visualized via incubation with di-aminobenzidine dihydrochloride. Slides were lightly counterstained with hematoxylin. Ki-67 positive stained cells were counted in five separate 400× microscopic fields per tumor sample.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick-End Labeling (TUNEL) Assay for Detection of Apoptosis *In Vivo*. Deparaffinized sections (5 μm) of tumor tissue were used to assess apoptosis using reagents supplied in the ApopTag *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Nuclei that stained brown were scored as positive for apoptosis, and those that stained blue were scored as negative. At least 16 microscopic fields (×400) were scored per tumor. Data are presented as the mean ± SE number of apoptotic cells counted in at least eight separate tumors from each group.

Statistical Analyses. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance (ANOVA) using SPSS (SPSS Inc., Chicago, IL). Difference in number of fluorescent microscopic metastases/group, Ki-67 stained cells/group, and TUNEL positive cells/group were determined using the two-tailed Mann-Whitney rank test using Prism software version 3.0 (Graphpad, San Diego, CA). A level of $P < 0.05$ was regarded as statistically significant.

Results

VES- and α-TEA-Inhibited DNA Synthesis in 66cl-4-GFP Cells *In Vitro*. 66cl-4-GFP cells were treated with VES or α-TEA to determine the ability of each to inhibit DNA synthesis (Fig. 1A). Cells treated with 2.5, 5, 10, or 20 μg/ml VES or α-TEA exhibited 9%, 14%, 30%, and 54% and 11%, 17%, 34%, and 82% reductions in DNA

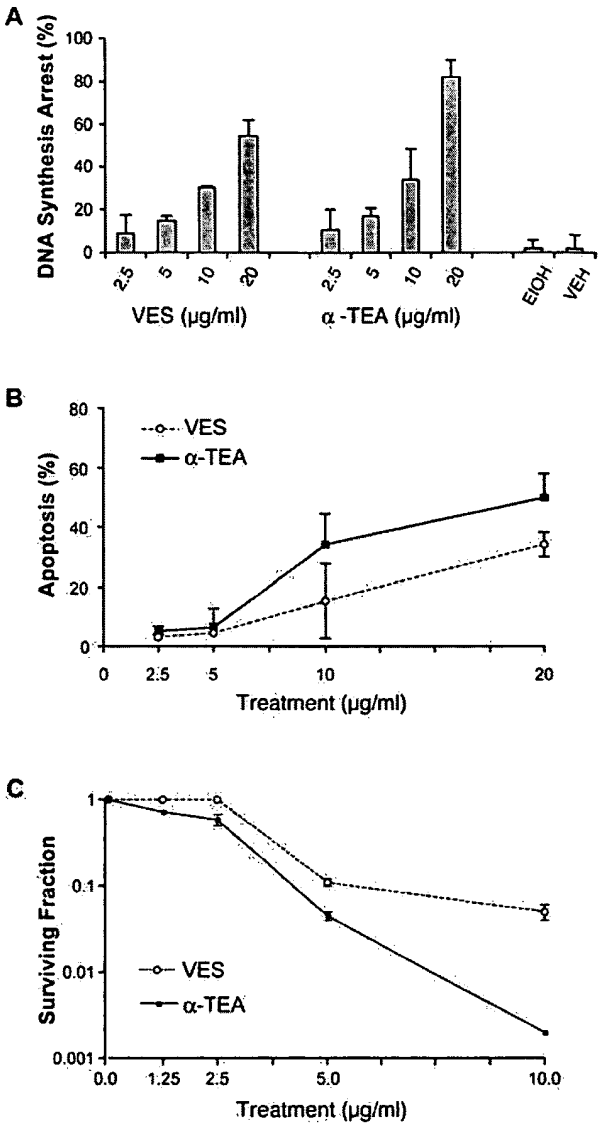

Figure 1. Documentation of VES and α-TEA induced DNA synthesis arrest and apoptosis and inhibition of colony formation. (A) Murine mammary cells were treated with varying concentrations of VES or α-TEA, equivalent amounts of ethanol (EtOH), or succinic acid + ethanol (VEH) in highest treatment dose or untreated and cultured for 24 hrs. Percent DNA synthesis arrest was determined by comparing the tritiated thymidine uptake (cpm) of treatment groups to tritiated thymidine uptake (cpm) of untreated controls. Data are mean ± SD of three independent experiments. (B) Murine mammary cancer cells were treated with varying concentrations of VES, α-TEA, or controls and cultured for 3 days. Apoptosis was measured by analyses of morphology of nuclei of DAPI-stained cells. Data are depicted as mean ± SD of three independent experiments. (C) Murine mammary cells were seeded at varying concentrations in tissue culture plates and treated with varying concentrations of VES or α-TEA for 10 days. Survival fractions were determined as number of colonies present divided by number of cells seeded × plating efficiency. Data are mean ± SD of two independent experiments.

synthesis after 24 hrs of treatment when compared with untreated controls, respectively (Fig. 1A). Vehicle (VEH) and ethanol (EtOH) controls exhibited 2% DNA synthesis arrest when compared with untreated control.

VES- and α-TEA-Induced Apoptosis in 66cl-4-GFP Cells, *In Vitro*. 66cl-4-GFP mammary cancer cells were treated with VES or α-TEA, and apoptosis was assessed by analyses of DAPI-stained cells for condensed nuclei and fragmented DNA. The level of apoptosis of 66cl-4-GFP cells treated for 3 days with 2.5, 5, 10, or 20 μg/ml VES or α-TEA was 3%, 5%, 16%, and 34%, and 5%, 6%, 34%, and 50% apoptosis, respectively (Fig. 1B). The levels of apoptosis induced by α-TEA and VES were not statistically different when compared pairwise by dose. However, percent apoptosis increased linearly with increasing dose of each agent, and linear regression analyses of the data showed that percent apoptosis increased more steeply with dose of α-TEA than dose of VES. An indicator-variable approach was used to test the null hypothesis of equal slopes, which was rejected ($P = 0.002$). Vehicle- and ethanol-treated controls exhibited 2%–3% apoptosis (data not shown).

VES and α-TEA Inhibited Colony Formation of 66cl-4-GFP Cells. In this study, the plating efficiency for 66cl-4-GFP cells varied between 24% and 40%. The surviving fraction of 66cl-4-GFP cells after treatment with 1.25, 2.5, 5, or 10 μg/ml of VES or α-TEA was 1.0 ± 0.0, 1.0 ± 0.0, 0.1 ± 0.01, and 0.05 ± 0.01 and 0.72 ± 0.01, 0.58 ± 0.12, 0.045 ± 0.007, and 0.002 ± 0.0 after 10 days of treatment, respectively (Fig. 1C).

Liposomal Formulations of VES and α-TEA Delivered by Aerosol Decreased 66cl-4-GFP Tumor Burden. Mean tumor volumes of control animals treated with liposomes by aerosol for 21 days were significantly higher than mean tumor volumes of animals treated by aerosol with liposomal formulations of either VES or α-TEA ($P < 0.001$; Fig. 2A). There were no differences in the mean tumor volumes between mice receiving the VES or α-TEA treatments (Fig. 2A). On the basis of previous experiments (12), we estimate that 36 μg of α-TEA or VES were deposited in the respiratory tract of each mouse each day. Thus, for the 21-day treatment period, we estimate that each mouse received 756 μg of α-TEA or VES from liposomal aerosol delivery.

Liposomal Formulations of α-TEA Delivered by Gavage but Not Liposomal Formulations of VES Delivered by Gavage Reduced 66cl-4-GFP Tumor Burden. Mean tumor volumes of mice treated by gavage with liposomal formulated α-TEA were significantly lower than that of control mice ($P < 0.001$; Fig. 2B). Vitamin E succinate incorporated into liposomes and delivered by gavage was not effective in reducing primary tumor burden when compared with control animals ($P < 0.10$; Fig. 2B). Although mice received 6.4 mg/day for 21 days of treatment by gavage for a total of 134.4 mg, we do not know the bioavailability of α-TEA or VES delivered by this method.

Liposomal Formulations of α-TEA and VES Delivered by Aerosol Suppressed 66cl-4-GFP Lung and Lymph Node Metastasis in BALB/c Mice. At sacrifice, all five lung lobes and axillary and brachial lymph nodes were examined visually for macroscopic metastases. The α-TEA aerosol treatment groups contained one animal (10%; total of one metastases) and the VES aerosol treatment group contained three animals (30%; total of five metastases) exhibiting macroscopic lung metastases, respectively, whereas the aerosol control group contained five animals (50%; total of 17 metastases) exhibiting macro-

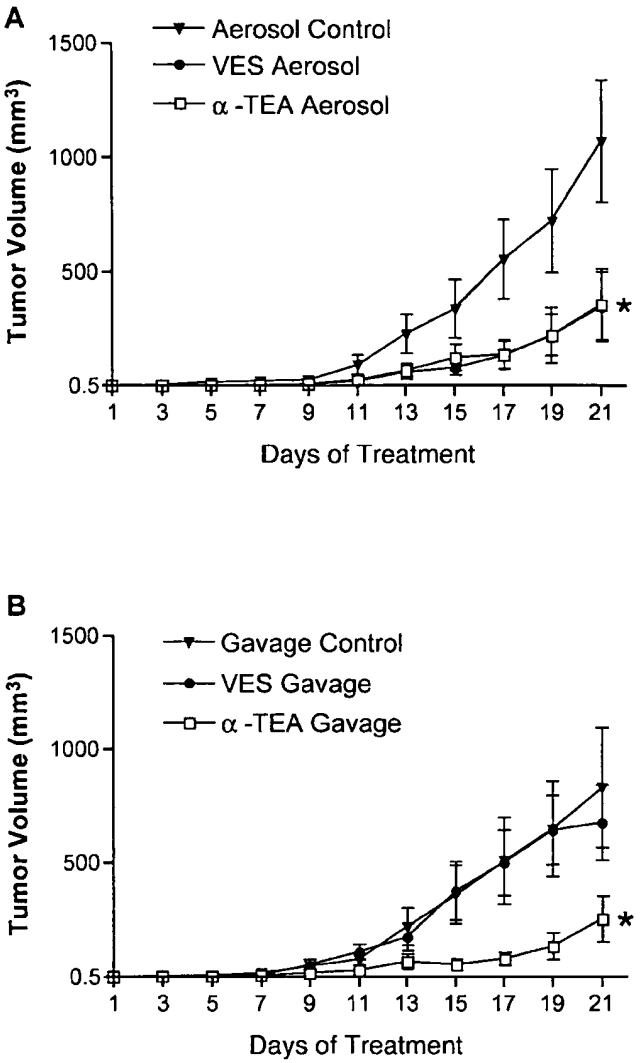

Figure 2. Comparisons of effects of liposomal formulated α-TEA or VES delivered by either aerosol (A) or gavage (B) on tumor burden. 66cl-4-GFP cells (2×10^5/mouse) were injected into the inguinal area at a point equidistant between the fourth and fifth nipples. Nine days after tumor cell injection, treatments were initiated and administered daily for a total of 21 days. Tumor volume/mouse was determined at 2-day intervals. Tumor volumes (mm^3) are depicted as mean ± SE. (A) Mice were treated with liposomal formulated α-TEA (80 mg/cage/day), VES (80 mg/cage/day), or liposome control by aerosol. (B) Mice were treated with liposomal formulated α-TEA (6.4 mg/mouse/day), VES (6.4 mg/mouse/day), or liposome control by gavage. * Designates a significant reduction in tumor burden in comparison with control ($P < 0.001$).

Table 1. 66cl-4-GFP Mammary Cancer Cell Macroscopic Lung Metastasis in BALBb/c Mice Receiving Liposomal Formulated α-TEA or VES by Aerosol or by Gavage

Delivery/Treatments	No. animals/group with macroscopic lung metastases[a]	Total no. macroscopic lung tumor foci[b]
Aerosol/liposomal control	5/10	17
Aerosol/liposomal VES	3/10	5
Aerosol/liposomal α-TEA	1/10	1
Gavage/liposomal control	7/10	12
Gavage/liposomal VES	5/10	7
Gavage/liposomal α-TEA	0/10	0

[a] Macroscopic lesions in all five lung lobes for each animal in all treatment groups were counted visually at the time of sacrifice.
[b] Data are expressed as the total number of macroscopic lung tumor foci observed in the 10 mice in each treatment group.

scopic lung metastases (Table 1). The number of mice with macroscopic metastases in the aerosol α-TEA and VES treatment groups was not significantly different from control or from each other, but in terms of total numbers of macroscopic lung tumor foci, the α-TEA group was significantly lower than control ($P < 0.048$). For oral treatments, both the number of mice with macroscopic lung metastases and the total number of tumor foci observed in the α-TEA treatment group were statistically lower than control or VES ($P < 0.003$ and $P < 0.03$; and $P < 0.02$ and $P < 0.015$, respectively). No macroscopic metastases were observed in the lymph nodes of any of the aerosol/liposomal groups: control, α-TEA, or VES (data not shown).

Use of a Nikon fluorescence microscope permitted measurement of green fluorescing microscopic metastases into three size groupings (small, <20 μm; medium, 20–50 μm; and large, >50 μm; Fig. 3A). Because the tumor cells are ~10–20 μm in diameter, the microscopic metastases scored as <20 μm most likely represent single cells. This analysis showed a decrease in microscopic lung metastases between the aerosol control group and both the α-TEA and VES aerosol treatment groups. The mean ± SE of total lung microscopic metastases in the α-TEA (31.2 ± 2.7; $n = 10$) and the VES (43.9 ± 5.3; $n = 10$) treatment groups in comparison to aerosol control (73.4 ± 8.8; $n = 10$) were significantly reduced ($P < 0.0001$ and $P < 0.009$, respectively; Fig. 3A). Of interest, lung microscopic metastases in the VES aerosol–treated animals were significantly lower than control in only one of the size categories of microscopic metastases; that is, the medium-sized lesions ($P < 0.007$), whereas the α-TEA aerosol–treated animals showed significant decreases in all three size categories. P values for α-TEA aerosol versus VES aerosol for mean number of small, medium, and large microscopic lung metastases are 0.02 versus 0.07, 0.0001 versus 0.007, and 0.0008 versus 0.15, respectively.

Mice treated with α-TEA aerosol had a lower number of axillary and brachial lymph node microscopic metastases,

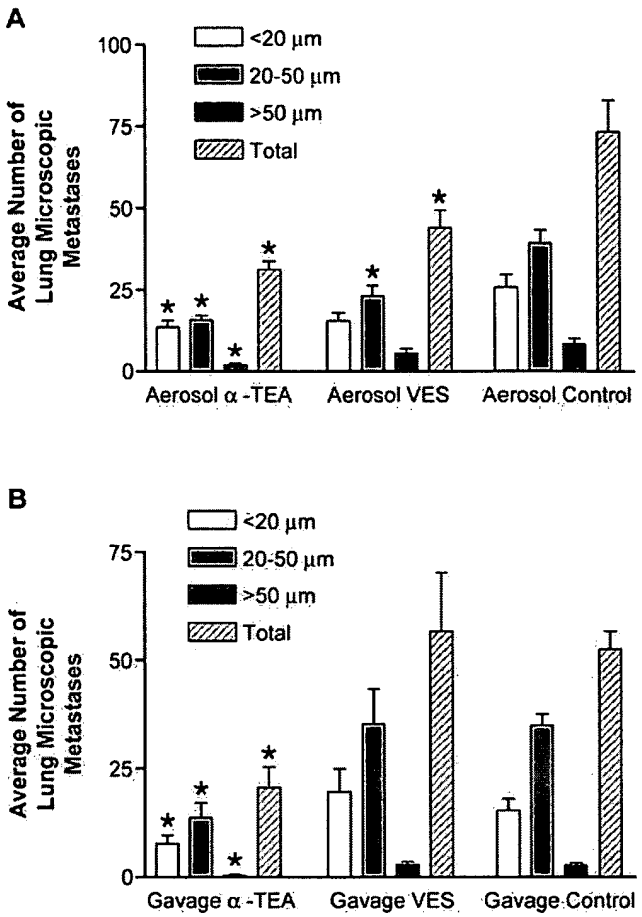

Figure 3. Comparison of effects of α-TEA or VES on lung microscopic metastases. The number of fluorescent microscopic metastases on the surface (top and bottom) of flattened left lung lobes from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or by gavage (B) were determined. Data are depicted as average ± SE of total number of lung microscopic metastases or number of metastases in each size category ($n = 10$ for each treatment group). * Designates a significant reduction in metastatic lesions in comparison with appropriate control.

whereas mice treated with VES aerosol showed no significant decrease in microscopic metastases found in lymph nodes (Fig. 4A). More specifically, α-TEA aerosol–treated mice had a mean ± SE of 1.4 ± 0.5 microscopic metastases per lymph node as compared with a mean ± SE of 6.0 ± 1.1 microscopic metastases found in lymph nodes from control animals ($P < 0.0001$). In contrast, VES aerosol–treated mice had a mean ± SE of 4.4 ± 1.2 microscopic metastases per lymph node in comparison with control mice ($P < 0.19$). Of additional interest, 48% of lymph nodes in mice treated with α-TEA aerosol were free of micrometastases, in comparison with 4% in control mice and 20% in VES aerosol–treated mice.

Liposomal Formulations of α-TEA Delivered by Gavage Suppressed 66cl-4-GFP Lung and Lymph Node Metastasis in BALB/c Mice, Whereas Liposomal Formulations of VES Delivered by Gavage

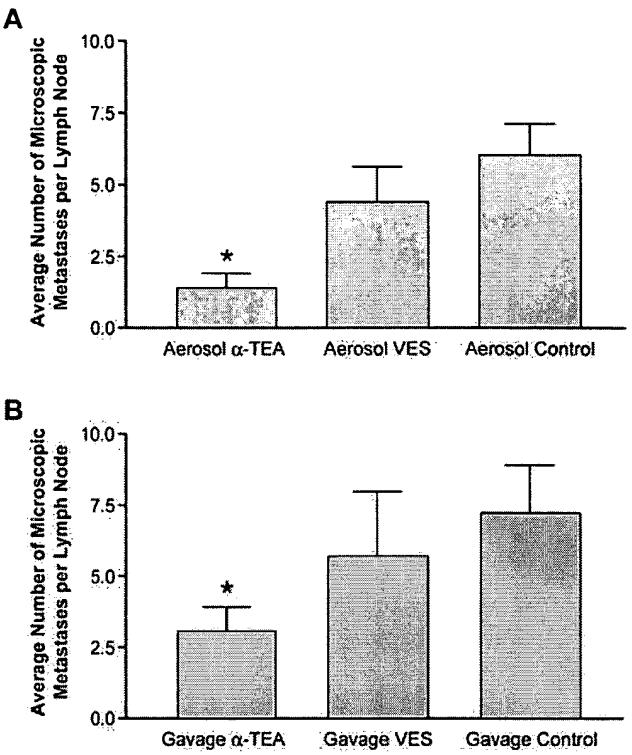

Figure 4. Comparison of effects of α-TEA or VES on microscopic metastases in lymph nodes. The number of fluorescent microscopic metastases on the surface of flattened axillary and brachial lymph nodes from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or by gavage (B) were determined. Number of lymph nodes/treatment group examined were aerosol α-TEA ($n = 21$), aerosol VES ($n = 15$), aerosol control ($n = 23$), gavage α-TEA ($n = 22$), gavage VES ($n = 22$), and gavage control ($n = 20$). Data are depicted as average ± SE of number of microscopic metastases/lymph node. * Designates a significant reduction in metastatic lesions compared with control.

Did Not. At sacrifice, all five lung lobes and axillary and brachial lymph nodes were examined visually for macroscopic metastases. There were no macroscopic lung metastases in the α-TEA gavage treatment group (0/10; 0%; 0 metastases), 5/10 (50%; total of 7 metastases) in the VES gavage treatment group, and 7/10 (70%; total of 12 metastases) in the gavage control group (Table 1). No macroscopic metastases were found in the lymph nodes of any animals from any of the gavage/liposomal treatment groups.

Use of a Nikon fluorescence microscope permitted measurement of green fluorescing microscopic metastases in lung tissue into three size groupings as discussed above. This analysis showed a decrease in the total number of microscopic lung metastases between the α-TEA gavage–treated mice, but not the VES gavage–treated mice in comparison with the gavage control animals. More specifically, the mean number of total microscopic metastases in the α-TEA gavage treatment group (21.5 ± 4.9; $n = 10$) and the VES gavage treatment group (57.4 ± 13.4; $n = 10$) in comparison with the gavage control group (52.7 ± 4.2; $n = 10$) was significantly reduced ($P < 0.0006$) for the former but not for the latter ($P < 0.74$; Fig. 3B). P values for α-TEA gavage versus VES gavage for mean number of small, medium, and large microscopic lung metastases are 0.05 versus 0.92, 0.0004 versus 0.49, and 0.0004 versus 0.90, respectively.

Mice treated with α-TEA by gavage had a lower number of microscopic metastases found in the axillary and brachial lymph nodes in comparison with control mice (Fig. 4B), whereas mice treated with VES by gavage exhibited no significant decrease in microscopic metastases found in lymph nodes in comparison with control mice (Fig. 4B). More specifically, α-TEA and VES gavage-treated mice had a mean ± SE of 3.0 ± 0.8 and 5.7 ± 2.2 microscopic metastases/lymph node; respectively, in comparison with a mean ± SE of 7.1 ± 1.7 microscopic metastases/lymph nodes in control animals ($P < .05$ and $P < 0.32$, respectively; Fig. 4B). Of additional interest, 32% of lymph nodes from mice treated with α-TEA by gavage were free of micrometastases, versus 23% in VES-treated and 15% in control mice.

Liposomal Formulations of VES or α-TEA Delivered by Aerosol or Gavage Did Not Exhibit Toxicity. No differences in mean body weights and no adverse side effects were found among any of the treatment or control groups (data not shown).

Inhibition of Cell Proliferation by VES and α-TEA *in Vivo*. Tumor sections from each of the treatment groups were examined by immunohistochemistry for proliferation status using the nuclear Ki-67 antigen expressed in proliferating cells as a biomarker. Tumors from mice treated with α-TEA via aerosol or gavage had mean ± SE of 151 ± 27.7 and 107.8 ± 27.2 Ki-67 positive cells/field, respectively, in comparison with tumors from aerosol and gavage control mice that had mean ± SE of 253.8 ± 34.7 and 241.1 ± 45.9 Ki-67 positive cells/field ($P < 0.05$ and $P < 0.03$, respectively; Fig. 5A and B). Ki-67 staining of tumors from mice treated with VES via aerosol or gavage showed no significant decrease in proliferation in comparison with tumors from corresponding control animals, with a mean ± SE of 167.8 ± 47.1 and 258.5 ± 26.8 Ki-67 positive cells/field ($P < 0.4$ and $P < 0.97$, respectively; Fig. 5A and B). There was no significant difference between VES and α-TEA in the mean number of Ki-67 positive cells observed when they were delivered by aerosol ($P < 0.96$).

Induction of Apoptosis by α-TEA and VES *in Vivo*. Tumors from treatment and control mice were taken at the completion of 21 days of treatment. Tumor sections from each of the treatment groups were examined by immunohistochemistry for apoptosis using TUNEL. No differences were found in the location of apoptotic cells in tumor sections from mice treated with α-TEA or VES delivered by aerosol or gavage. Tumors from mice treated with α-TEA delivered by aerosol or gavage had a mean ± SE of 1.54 ± 0.37 and 1.31 ± 0.31 apoptotic cells/field, respectively, whereas tumors from aerosol and gavage

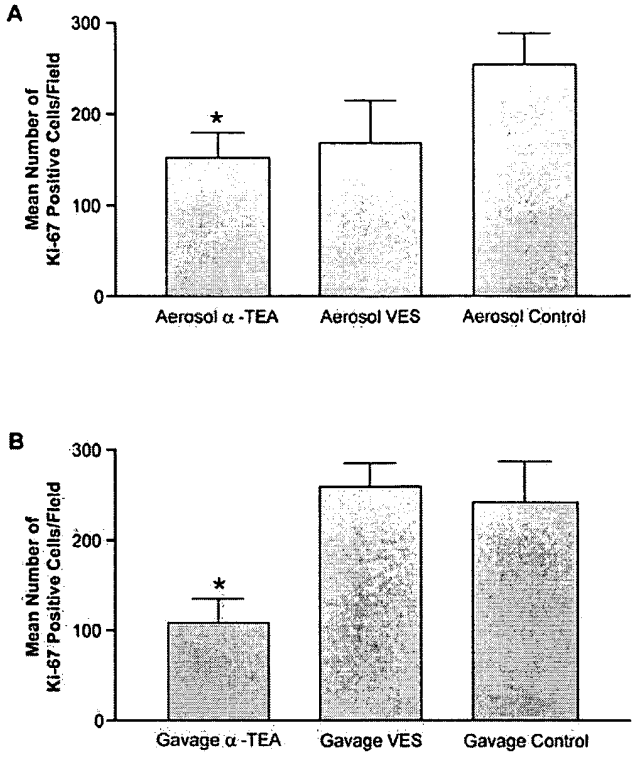

Figure 5. α-TEA, but not VES, significantly inhibited primary tumor cell proliferation. Comparisons of Ki-67 positive cells in tumor sections (5 μm) obtained from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or by gavage (B) are depicted. Number of tumors/treatment group examined were aerosol α-TEA (n = 8), aerosol VES (n = 8), aerosol control (n = 9), gavage α-TEA (n = 8), gavage VES (n = 8), and gavage control (n = 9). Five separate sections of each slide/tumor were scored for Ki-67 positive cells. Data are depicted as mean ± SE Ki-67 positive cells/field. * Designates a significant reduction in proliferating cells in comparison with control.

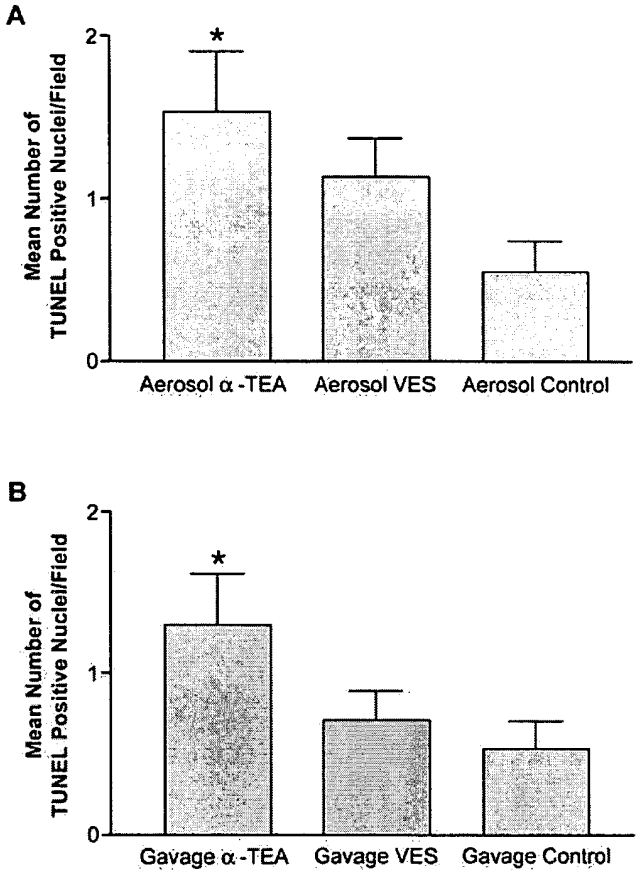

Figure 6. α-TEA, but not VES, significantly induced apoptosis. Comparisons of deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL)-positive nuclei in tumor sections (5 μm) obtained from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or delivered by gavage (B) are given. Number of tumors/treatment group examined were aerosol α-TEA (n = 8), aerosol VES (n = 8), aerosol control (n = 9), gavage α-TEA (n = 8), gavage VES (n = 8), and gavage control (n = 9). The number of TUNEL-positive nuclei were scored in 16 microscopic fields (×400)/tumor. The data are depicted as the mean ± SE of number of TUNEL-positive nuclei/ field. * Designates a significant increase in apoptotic cells in comparison to control.

control mice had a mean ± SE of 0.55 ± 0.19 and 0.54 ± 0.17 apoptotic cells/field, respectively ($P < 0.03$ and $P < 0.05$; Fig. 6A and B). Tumors from mice treated with VES delivered by aerosol or gavage exhibited increased numbers of apoptotic cells but showed no significant increase in apoptotic cells in comparison with control animals, that is, mean ± SE of 1.14 ± 0.23 and 0.71 ± 0.18 apoptotic cells/field ($P < 0.09$ and $P < 0.70$, respectively; Fig. 6A and B).

Discussion

The goal of the studies reported here was to compare the antitumor properties of two vitamin E derivatives *in vitro* and *in vivo* when both compounds were formulated into liposomes and delivered either by aerosol or orally by gavage. Cell culture studies reported here showed that both vitamin E derivatives were effective antitumor agents, capable of inducing dose-dependent DNA synthesis arrest and cell death by apoptosis as well as inhibiting colony formation of murine mammary cancer cells.

In vivo, both α-TEA and VES significantly decreased primary tumor burden when delivered by aerosol. The effectiveness of VES delivered by aerosol is a novel finding because previous studies of antitumor effects of VES in animal xenograft and allograft models administered VES intraperitoneally (12, 22–26). Although both vitamin E derivatives when delivered by aerosol were effective in reducing metastasis, α-TEA decreased lung and lymph node metastasis to a greater degree than VES.

α-TEA was effective when delivered orally; however, oral delivery of VES was ineffective. The ineffectiveness of oral VES delivery was expected based on *in vitro* analyses demonstrating a need for the intact compound for antitumor activities (19, 27–29), and potential for de-esterification by intestinal esterases (30, 31).

A comparison of the tumor efficacy of α-TEA and VES liposomal formulations delivered by aerosol versus gavage showed α-TEA to be effective when delivered by either route, whereas VES was effective only when delivered by

aerosol. As mentioned above, the factor most likely contributing to this difference is that esterases in the intestinal tract most likely cleaved the ester-linked succinic acid moiety of VES, producing free succinic acid and RRR-α-tocopherol, neither of which exhibits anticancer properties (12). Other possible reasons for the difference in efficacy between aerosol and oral delivery include differences in particle size (2.0 μm for aerosol versus 4–10 μm for gavage), which might affect bioavailability; differences in total amounts administered; and differences in the role a liver vitamin E–binding protein, α-tocopherol transfer protein (α-TTP) that selectively incorporates natural vitamin E (RRR-α-tocopherol) from the diet, might play in bioavailability.

There do not appear to be any stability differences between α-TEA and VES liposomal preparations. On the basis of our limited studies with liposomal α-TEA and liposomal VES, lyophilized preparations appear to be stable over several weeks. We did not see any difference in the liposomal-formulated compounds before or after 15 mins of nebulization. Lyophilized α-TEA and VES were resuspended in water, and formation of liposomes and possibly crystals was observed by light microscopy with a polarizing filter to visualize crystals. After aerosolization, the material remaining in the reservoir of the nebulizer was again visualized. No crystals were observed in either the α-TEA or VES liposomal formulations before or after aerosolization. Aerosol concentrations of α-TEA or VES over the 15-min nebulization period gave a similar pattern. High-performance liquid chromatography (HPLC) and UV analyses showed the retention time and area under the peak curve for α-TEA and VES to be similar before and throughout aerosolization. These observations suggest that there were no chemical or physical alterations of α-TEA or VES during the aerosolization process. In summary, lyophilized formulations of α-TEA and VES appear to be stable for several weeks and, once resuspended in water, the liposomal formulations appear to be very stable before and after aerosolization.

Vitamin E compounds are relatively nontoxic and are extremely well tolerated by humans, with reported side effects being of a relatively minor nature, primarily gastrointestinal symptoms, generalized dermatitis, and fatigue in a subset of subjects (32). The Food and Nutrition Board has established the Tolerable Upper Intake Level for vitamin E to be 1000 mg/day for healthy adults aged 19 to 70 (33). Regarding the safety of VES and α-TEA, limited studies in mice suggest that they are also relatively nontoxic because studies have failed to achieve an oral LD50 dose, and daily administration (either orally or via aerosolization) for up to 36 days has not produced any overt signs of toxicity such as weight loss or observable changes in behavior. More studies need to be conducted.

Findings reported here suggest that liposomal formulations of vitamin E derivatives may be beneficial to antitumor efficacy. Regarding oral administration, it is important to note that previous studies reported that α-TEA was not effective in reducing tumor burden when delivered orally in a peanut oil suspension (12). Thus, the positive results achieved here are likely due to liposomal formulation as well as increased dosage achieved by twice-a-day gavaging.

Data showing that α-TEA administered by aerosol inhibited microscopic metastases in lymph nodes is noteworthy because a high percentage of lymph nodes in the α-TEA treatment group did not show any metastases. In comparison, when VES was administered via aerosol, it was effective in significantly reducing total number of microscopic metastases in the lungs, but it it was ineffective in significantly reducing lymph node metastasis. This observation may reflect tissue uptake differences between VES and α-TEA or perhaps tissue-specific inactivation of VES by esterase cleavage. Future studies will be required to understand this difference.

Analyses of cell proliferation and apoptosis by staining tumor sections for the nuclear Ki-67 antigen that is a biomarker for proliferating cells, and staining for TUNEL that is a biomarker for apoptosis, suggested that both α-TEA (aerosol and oral) and VES (aerosol only) reduced tumor burden by decreasing cell proliferation and increasing apoptosis. This correlates directly with mechanisms documented in cell culture studies. Although the ability to decrease cell proliferation and increase apoptosis *in vivo* in comparison with controls was statistically significant for α-TEA only, it is important to point out that no significant differences were detected between the ability of α-TEA (aerosol) and VES (aerosol) to decrease cell proliferation.

In summary, data reported here are promising in that they show that a novel vitamin E analog, α-TEA, when formulated into liposomes and administered either by aerosol or orally, has the ability to decrease primary tumor burden and reduce lung and lymph node metastasis in a syngeneic tumor model. This positive antitumor activity occurred without any overt toxic effects. In comparison, the vitamin E derivative, VES, although effective at decreasing primary tumor burden when formulated into liposomes and delivered via aerosol, was less effective at inhibiting metastasis and had no antitumor properties when delivered orally. These studies support the further development of α-TEA as a potential chemotherapeutic agent.

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Report

Vitamin E analog α-TEA and celecoxib alone and together reduce human MDA-MB-435-FL-GFP breast cancer burden and metastasis in nude mice

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Key words: apoptosis, CD-31, celecoxib, human MDA-MB-435-FL-GFP breast cancer xenografts, Ki-67 nuclear antigen, metastasis, TUNEL, vitamin E analog α-TEA

Summary

α-TEA, a nonhydrolyzable ether analog of vitamin E (RRR-α-tocopherol), and celecoxib, a specific COX-2 inhibitor, were delivered separately or in combination to investigate their anticancer properties, using MDA-MB-435-FL-GFP human breast cancer xenografts in nude mice. Liposomal formulated α-TEA administered as an aerosol and celecoxib fed at 500 or 1250 mg/kg diet for 31 days separately or in combination significantly reduced tumor volume in comparison to control ($p < 0.001$ for all treatment groups). Of special note, the combinations of α-TEA + celecoxib (1250) inhibited tumor volume significantly better than either single treatment ($p < 0.001$ and $p < 0.001$). Average number of macroscopic lung metastases were significantly reduced in all treatment groups in comparison to control, with the exception of celecoxib (500). Mean numbers of microscopic lung and lymph node metastases in all treatment groups were significantly lower than control. Furthermore, the mean number of microscopic lung metastases in the α-TEA + celecoxib (1250) group were significantly lower than either separate treatment. Analyses of 5 μm tumor sections showed that all treatments, with the exception of celecoxib (500) alone, significantly enhanced apoptosis (TUNEL) and significantly decreased cell proliferation (Ki-67). α-TEA and α-TEA + celecoxib (1250) treatments significantly reduced blood vessel density (CD-31) in comparison to control. These data show promise for combination α-TEA + celecoxib chemotherapy for breast cancer.

Abbreviations: α-TEA: 2,5,7,8-tetramethyl-2R-(4R, 8R-12-trimethyltridecyl) chroman-6-yloxyacetic acid; COX-2: cyclooxygenase-2; MDA-MB-435-FL-GFP cells: high lung metastatic human estrogen nonresponsive breast cancer cells transfected with green fluorescent protein; JNK: c-Jun-N-terminal kinase; MAPK: mitogen activated protein kinase; TGF-β: transforming growth factor-beta; TUNEL: terminal deoxynucleotidyl transferase-mediated nick end labeling assay

Introduction

Breast cancer is the most common cancer among US women, and despite intense study, breast cancer remains the second leading cause of cancer deaths in American women [1]. Such statistics highlight the need for new drug development and alternate treatment strategies. Toxic effects of chemotherapeutic drugs on normal tissues focus the need for development of low toxicity drugs with potent anticancer properties, and strategies for combinations of low toxicity drugs exhibiting combined anticancer effects greater than singly administered drugs.

Our lab has developed one such drug, a stable, nonhydrolyzable acetic acid derivative of vitamin E (RRR-α-tocopherol), referred to as alpha-tocopherol ether analogue (α-TEA) [2]. The parent compound for making α-TEA is natural vitamin E (RRR-α-tocopherol). α-TEA has an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage yielding a stable, nonhydrolyzable entity [2].

Since α-TEA is a lipid which complicates i.v. administration and because there is a specific RRR-α-tocopherol transfer protein in the liver that selectively mediates the transfer of RRR-α-tocopherol into lipoproteins thus limiting bioavailability following oral administration of vitamin E compounds other than RRR-α-tocopherol, we are investigating the formulation of α-TEA into liposomes and aerosol delivery as a potential clinically-relevant cancer treatment strategy. In preclinical syngeneic transplantable mouse mammary cancer studies, α-TEA formulated into liposomes and delivered by aerosol has been shown to reduce tumor burden and inhibit metastasis to lungs [2]. Equally important, *in vivo* mouse mammary cancer syngeneic studies show α-TEA to have no adverse side effects [2]. *In vitro* mechanistic studies show α-TEA to sensitize human breast cancer cells to both Fas and TGF-β apoptotic signaling that converge on mitogen activated protein kinase (MAPK) c-Jun-N-terminal kinase (JNK) [3]. Thus, based on α-TEA's ability to induce tumor cells in culture to undergo apoptosis, and its anticancer effectiveness *in vivo* and lack of overt toxicity, we hypothesize that α-TEA, administered separately or in combination with other drugs, may show promise for the prevention and treatment of breast cancer.

Cyclooxygenase-2 (COX-2) is a promising molecular target best studied in colorectal cancers, more recently in breast cancer [4–12]. COX-2 is expressed constitutively in many cancers, but not normal cells. COX-2 serves as a selective target for tumor cells and has been implicated in promoting cancer growth, causing neoplastic transformation, and enhancing angiogenesis and invasiveness [11, 13–19]. COX-2 inhibitors have shown promise in the prevention of carcinogen-induced mammary cancer in rats [5, 6, 9], chemoprevention studies of mammary cancer in transgenic mice [10–12], syngeneic mouse mammary cancer studies [8], and studies of human breast cancer xenografts in immune compromised mice [7]. Celecoxib, is a selective inhibitor of COX-2 that has been approved for evaluation in preventing sporadic colorectal adenomas [30].

Studies of α-TEA in our lab have shown α-TEA to induce apoptosis by converting transforming growth factor-beta (TGF-β) and Fas (CD95)/Fas ligand resistant human breast cancer cells to TGF-β and Fas responsive cells, and signaling apoptosis *via* the mitogen activated kinase JNK [3, 20]. Since the anticancer effects of celecoxib appear to differ from α-TEA, studies were conducted to determine whether treatments with the combination of α-TEA + celecoxib would reduce tumor burden and metastasis of MDA-MB-435-FL-GFP breast cancer cells transplanted into immune compromised mice greater than each agent tested separately.

Materials and methods

Chemicals and reagents

α-TEA was prepared as described previously [2]. Celecoxib (SC-58635; 4-[5-(4-methylphenyl)-3-trifluoromethyl-1H-pyrazol-l-yl] benzenze-sulfomamide) was purchased from LKT Laboratories (St Paul, MN).

Immune compromised nude mice

Female out bred NU/NU immunodeficient mice 4–6 weeks of age were used for the study. They were purchased from Charles River Laboratory Inc. (Wilmington, MA), and maintained in a sterile environment in the transgenic facilities at the Animal Resource Center at the University of Texas at Austin at 74 ± 2 °F with 30–70% humidity and a 12 h alternation light–dark cycle. The animals accessed water and powdered AIN-76A diet (Dyets, Inc. Bethlehem, PA) *ad libitum*. Sixty mice were used for the study, (10/group).

Human MDA-MB-435-FL-GFP breast cancer cell line

MDA-MB-435-FL-GFP human breast cancer cell line was generated from lung metastases of MDA-MB-435-GFP cells that were inoculated subcutaneously in the flank of a female athymic nude

mouse. It was certified to be pathogen free. MDA-MB-435-GFP cells were obtained after stable transfection of the parental MDA-MB-435 cells with an expression vector of the enhanced green fluorescence protein (GFP) (pEGFP-N1 from Clontech Laboratories, Inc.). MDA-MB-435-FL-GFP cells were maintained as monolayer cultures in McCoy's 5A media (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), 1.5 mM sodium pyruvate (Sigma Chemical Co.), 100 mM streptomycin, 100 IU/ml penicillin (Gibco/BRL), 1× (v/v) nonessential amino acids (Sigma), 1× (v/v) MEM vitamins, and 50 μg/ml gentamycin (Sigma).

Supplementation of AIN76A diet with celecoxib

Powdered celecoxib (500 or 1250 mg/kg) was mixed with AIN-76A powdered diet for 30 min using a blender. Diets with celecoxib supplementation were prepared every week, and stored at 4 °C until used. Diet was replaced every 2 days. Food consumption for control and all treatment groups was recorded every 2 days at the time when food was replaced.

Preparation of α-TEA liposomal formulation for delivery by aerosol

An α-TEA liposome ratio of 1:3 (w/w) was previously determined to be optimal [2]. Briefly, DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine; Avanti Polar-Lipids, Inc., Alabaster, AL) at 120 mg/ml was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX) and brought to a clear solution by sonication. α-TEA at 40 mg/ml was dissolved in tertiary-butanol and dissolved by vortexing. Equal amounts (v:v) of DLPC and α-TEA were combined to yield a 1:3 ratio of α-TEA/liposome, mixed by vortexing, frozen at −80 °C for 1–2 h, and lyophilized overnight to a dry powder prior to storing at −20 °C until needed.

Aerosol delivery of α-TEA liposome preparation

Liposome control and liposomal α-TEA were administered by aerosol, as previously described [2, 21, 22]. Briefly, aerosol was generated using an Easy Air 15 Compressor (Precision Medical, Northampton, PA) producing a 10 l/min airflow with an AeroTech II nebulizer (CIS-US, Inc., Bedford, MA). Liposome particles of a mass median aerodynamic diameter of 2.01 μm with a geometric SD of 2.04 were determined as previously described [2, 22]. Mice were placed in plastic cages (7 × 11 × 5 in.) with a sealed top. Aerosolization was conducted in a safety hood. Aerosol entered the cage *via* a 1-cm accordion tube at one end and was discharged at the opposite end, using a one-way pressure release valve. Mice were exposed to aerosol until all liposomal α-TEA or liposome only (control) was aerosolized (~15 min). As previously determined, we estimate that 36 μg of α-TEA were deposited in the respiratory tract of each mouse each day. Thus, for the 31-day treatment period, we estimate that each mouse received 1.116 mg of α-TEA from aerosol delivery [2].

Treatment groups

Mice were injected subcutaneously in the inguinal area at a point equal distance between the fourth and fifth nipples on the right side with 1×10^6 MDA-MB-435-FL-GFP cells (100 μl volume) using a 25-gauge needle. Ten days after tumor cell inoculation (tumor volumes approximately 14 mm^3), tumor bearing mice were randomly divided into control and five treatment groups of 10 mice/group (α-TEA, celecoxib (500 or 1250 mg/kg diet), and combinations of α-TEA + celecoxib (500 or 1250 mg/kg diet). Control mice received liposome only by aerosol. α-TEA was formulated in liposomes and delivered by aerosol (75 mg/cage/day); diets were supplemented with celecoxib (500 or 1250 mg/kg diet). All mice were fed *ad libitum* using powdered AIN-76A diet, and all treatments were daily for 31 days.

Measurement of body weight and tumor volume

Body weights, activity, and appearance of mice were recorded at weekly intervals. Tumor growth was measured by caliper evaluations of tumor size every other day. Tumor volume was calculated according to the equation $V = (L \times W^2)/2$, where V is the volume of each tumor, L is the length, and W is the width [23].

Determination of lung and lymph node metastasis

At sacrifice, visual macroscopic lung metastases were counted, brachial and axillary lymph nodes, and the left lung lobe were taken for analyses of fluorescent green microscopic metastases. (Note: Since the lungs of mice are not symmetrical, the left lung which consists of one large lobe but not the right lung which consists of four lobes was used.) Lung lobes and lymph nodes were flattened and the entire surface (both sides for lung and one side only for lymph nodes) of each of the tissues were scored for fluorescent green microscopic metastases using a Nikon fluorescence microscope (TE-200; 200× magnification) equipped with an ocular grid for use in size determination of metastases. Fluorescent microscopic lung metastases were placed into four size groupings: <20, 20–50, 50–100, and >100 μm. On the basis of a typical MDA-MB-435-FL-GFP breast cancer cell size of 10–20 μm in diameter, the <20-μm grouping is thought to represent solitary cells; the 20–50 μm grouping 2–5 cells; the 50–100 μm grouping 5–10 cells; and the >100 μm grouping >10 cells. The fluorescent microscopic lymph node metastases were also placed into these same four size groupings plus a group referred to as "patches" which contained between 100 and 200 cells.

Ki-67 staining for detection of proliferation in vivo

Deparaffinized 5 μm tumor sections from five mice in control and treatment groups were examined using antibody specific for nuclear antigen Ki-67, a biomarker for determining the number of cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked with 3% H_2O_2 and nonspecific antibody binding was blocked with 10% normal rabbit serum. Sections (5 μm) were incubated with Ki-67 antibody (anti-human Ki-67 antibody; DAKO Corp., Carpinteria, CA; 1:200 dilution) overnight at 4 °C; next, the tissue sections were incubated with biotinylated rabbit–anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 min at room temperature. Tissue sections were then incubated with avidin-biotin complex (ABC-HRP, Vector Laboratories) for 30 min at room temperature. Immunoreactivity was visualized *via* incubation with di-aminobenzidine dihydrochloride. Slides were lightly counterstained with hematoxylin. Ki-67 positive stained cells were counted in sixteen or more 400× microscopic fields per tumor sample. Data are presented as the mean ± SE.

TUNEL assay for detection of apoptosis in vivo

Deparaffinized 5 μm tumor sections were assessed for apoptosis using reagents supplied in the ApopTag *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Brown stained nuclei were scored as positive for apoptosis. Sixteen or more microscopic fields (400×) were scored/tumor. Data are presented as the mean ± SE number of apoptotic cells counted in five separate tumors from each group..2

CD31 staining for detection of blood vessel density

CD31 staining of 5 μm sections was used for the detection of intratumoral microvessel density based on the "hot spots" method [24]. Briefly, areas of highest neovascularization were found by scanning the tumor sections at low power (200×) with a light microscope. Areas with the greatest density of CD31 staining for control and treatment groups were selected, and microvessel counts were made using a 400× field. A total of 10 fields (400×) were counted. Data are presented as the mean ± SE.

Statistical analyses

Body weight and food consumption data were analyzed by *t*-test. Tumor growth was evaluated using a nested two factor ANOVA with Tukey HSD, follow-up comparisons were used to examine changes in logarithmic transformed tumor volume (base 10) between control and treatment groups. The differences in the number of macroscopic and microscopic metastatic lesions, Ki-67 positive cells, TUNEL positive cells, and CD31 positive cells were analyzed by *t*-test after logarithmic transformation (base 10) using SPSS (SPSS, Inc., Chicago, IL). A level of $p < 0.05$ was regarded as statistically significant.

Results

α-TEA and celecoxib separately or in combination suppressed MDA-MB-435-FL-GFP tumor growth in nude mice

Mean tumor volumes in both combination treatments were significantly lower than control ($p < 0.001$ for both); however, only the α-TEA + celecoxib (1250) combination was significantly lower than α-TEA or celecoxib (1250) administered alone ($p < 0.001$; Figure 1). Mean tumor volumes of α-TEA or celecoxib (500 or 1250 doses) were significantly lower than control ($p < 0.001$ for all three treatments, Figure 1).

α-TEA or celecoxib separately or in combination had no effect on body weights or food consumption

Control and all treatment groups gained weight. Compared with the control group, there were no significant differences in the body weight gains or food consumption over the experimental period in the α-TEA, celecoxib, and combination treatment groups (data not shown).

α-TEA or celecoxib separately or in combination suppressed visible macroscopic lung metastasis

At the completion of the study all five lung lobes from each mouse were examined for visible metastasis. The average number of macroscopic lung tumor foci were significantly reduced in all treatment groups, except the celecoxib (500) group, when compared to control (Table 1; α-TEA $p < 0.026$; celecoxib (1250) $p < 0.020$; α-TEA + celecoxib (500) $p < 0.022$; α-TEA + celecoxib (1250) $p < 0.001$). There were animals in each group that had no visible macroscopic metastatic lung lesions: two in control, six in α-TEA, three each in celecoxib 500 and celecoxib 1250, five in combination α-TEA + celecoxib (1250), and two in combinatin α-TEA + celecoxib (500) (Table 1). None of these groups differed statistically.

α-TEA and celecoxib separately or in combination suppressed microscopic lung metastasis

Green fluorescing microscopic lung metastases were classified into four size groups : <20, 20–50, 50–100, and >100 μm (Figure 2(a)). The mean total number of microscopic lung lesions in

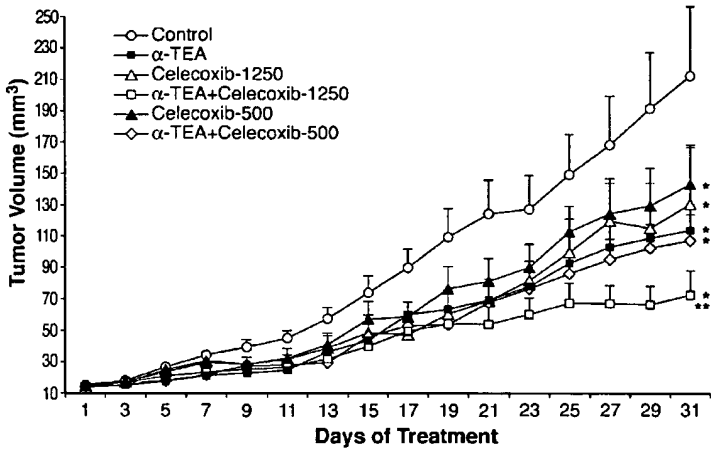

Figure 1. α-TEA and celecoxib reduced tumor growth when administered separately or together. Human MDA-MB-435 breast cancer cells at 1×10^6 cells/mouse were injected into the inguinal area of immune compromised NU/NU mice at a point equal distance between the fourth and fifth nipples. Ten days after tumor injection, mice were placed into six groups of 10 mice/group: control (–○–), α-TEA (–■–), celecoxib (1250 mg/kg diet) (–△–), α-TEA + celecoxib (1250 mg/kg diet) (–□–), celecoxib (500 mg/kg diet) (–▲–), and α-TEA + celecoxib (500 mg/kg diet) (–◇–). Liposome formulated α-TEA was delivered by aerosol and celecoxib was delivered in the diet. All mice were fed a powdered AIN-76A diet, and mice were treated daily for 31 days. Tumor volume/mouse was determined at 2-day intervals. Tumor volumes (mm^3) are depicted as mean ± SE. (*) = significantly different from control, (**) = significantly different from all other treatment groups.

Table 1. α-TEA and celecoxib treatments alone or together reduce visible macroscopic lung metastases

Treatments[a]	# Animals/ group with macroscopic lung metastases[b]	Average # macroscopic lung tumor foci[c]
Control	8/10	3.9 ± 0.6
α-TEA	4/10	1.5 ± 0.3*
Celecoxib (1250)	7/10	1.6 ± 0.2*
α-TEA + celecoxib (1250)	5/10	1.1 ± 0.1*
Celecoxib (500)	7/10	2.6 ± 0.3
α-TEA + celecoxib (500)	8/10	1.6 ± 0.3*

* Designates significant difference from control.
[a] α-TEA was formulated into liposomes and delivered daily (7 days/week) by aerosol, celecoxib was fed in the diet.
[b] Macroscopic lesions in all lung lobes for every animal in control and treatment groups were counted visually at the time of sacrifice.
[c] Data are expressed as the average number of macroscopic lung tumor foci observed in tumor bearing mice in control and treatment groups.

α-TEA, celecoxib (1250), α-TEA + celecoxib (1250), celecoxib (500), and α-TEA + celecoxib (500) treatment groups were significantly reduced in comparison to control (Figure 2(b); $p < 0.001$; $p < 0.001$, 0.001, 0.005, and 0.001, respectively). These significant reductions appear to be attributable mainly to lesions in the <20 μm size grouping (Figure 2(b)). The mean total number of microscopic lung lesions in the α-TEA + celecoxib (1250) combination group were significantly different from each of the individual treatments (Figure 2(b); $p < 0.033$ and 0.003, respectively). Again, these significant reductions appear to be attributable to the <20 μm size grouping but can also be observed in the 20–50 μm size grouping (Figure 2(b)).

α-TEA and celecoxib separately or in combination reduced microscopic lymph node metastasis

Green fluorescent microscopic lymph node metastatic lesions, like the lung microscopic lesions, were classified into four different size groups: <20, 20–50, 50–100, and >100 μm. "Patches" composed of 100–200 tumor cells were also observed. The mean total number of microscopic lymph node lesions in the α-TEA, celecoxib (1250), α-TEA + celecoxib (1250), celecoxib (500), and α-TEA + celecoxib (500) treatment groups were significantly reduced in comparison to control (Figure 3; $p < 0.017$; $p < 0.001$, 0.002, 0.008, and 0.001, respectively). These reductions appear to be primarily due to the 20 and 20–50 μm size lesions. The mean total number of microscopic lymph node lesions in the α-TEA + celecoxib (1250) combination group were significantly different from the mean total number in the α-TEA only treatment group ($p < 0.046$), but not the celecoxib (1250) only treatment group (Figure 3).

α-TEA and celecoxib separately or in combination induced tumor cells to undergo apoptosis in vivo

Apoptosis was evaluated using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining of 5 μm tumor sections from five mice each from control and all treatment groups. The mean number of TUNEL positive cells in all treatment groups with the exception of celecoxib (500) were significantly higher than control (Figure 4(a); α-TEA $p < 0.019$; celecoxib (1250) $p < 0.001$; α-TEA + celecoxib (1250) $p < 0.001$; and α-TEA + celecoxib (500) $p < 0.001$; respectively). The α-TEA + celecoxib (1250) combination treatment did not induce apoptosis significantly better than the single treatments alone (Figure 4(a)).

α-TEA and celecoxib separately or in *combination reduced tumor cell proliferation* in vivo

The mean number of proliferating cells determined by Ki-67 staining in all treatment groups, with the exception of celecoxib (500), showed significantly fewer proliferating cells in comparison to control (Figure 4(b); α-TEA $p < 0.031$; celecoxib (1250) $p < 0.006$; α-TEA + celecoxib (1250) $p < 0.003$; and α-TEA + celecoxib (500) $p < 0.015$; respectively). The α-TEA + celecoxib (1250) combination treatment did not block proliferation significantly better than the single treatments alone (Figure 4(b)).

α-TEA and celecoxib separately or in combination suppressed microvessel formation in tumors in vivo

Intratumoral microvessel density was evaluated using CD31 (PECAM-1) endothelial staining of 5 μm tumor sections from five mice each from

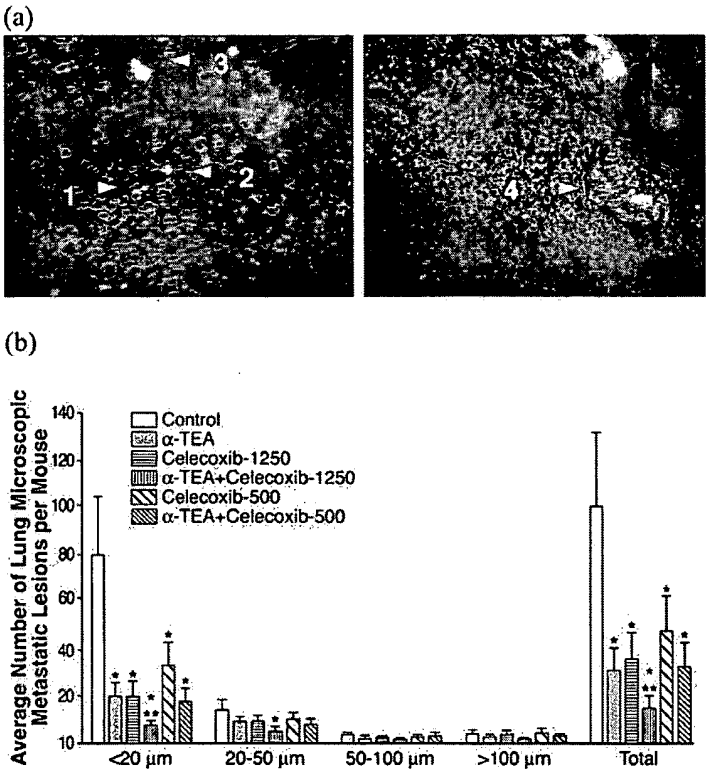

Figure 2. α-TEA and celecoxib reduced metastasis to the lung when administered separately or together. The left lung lobe from control and treatment groups was flattened and both sides were examined for fluorescent microscopic lesions. (a) Image of fluorescent lung lesions as viewed using a Nikon TE-200 fluorescent microscope. Numbered arrows designate the four size groupings that the lesions were placed into: (1) < 20 μm, (2) 20–50 μm, (3) 50–100 μm, and (4) >100 μm. (b) Average number of lung microscopic metastatic lesions per mouse for control and treated groups: Control (▭), α-TEA (▭), celecoxib (1250 mg/kg diet) (▭), α-TEA + celecoxib (1250 mg/kg diet) (▭), celecoxib (500 mg/kg diet) (▭), and α-TEA + celecoxib (500 mg/kg diet) (▭) Data are depicted as mean ± SE. (*) = significantly different from control, (**) = significantly different from α-TEA or celecoxib (1250 mg/kg diet) administered separately.

control or treatment groups. The numbers of microvessels were reduced in all treatment groups in comparison to control, but only the α-TEA alone and the α-TEA + celecoxib (1250) combination treatments were significantly reduced (Figure 4(c); $p < 0.015$ and 0.020, respectively).

Discussion

In this study we compared two levels of celecoxib (500 and 1250 mg/kg/diet) fed by diet separately and in combination with a liposomal formulation of the vitamin E analog α-TEA administered by aerosol in a human breast cancer xenograft model in order to determine if combined treatments would show significantly better antitumor efficacy.

These studies showed that all treatments significantly reduced tumor burden in comparison to control and that the combination of α-TEA + celecoxib (1250) treatment group reduced tumor burden significantly better than either α-TEA or celecoxib treatment alone. Furthermore, all treatment groups, with the exception of celecoxib (500) alone, significantly reduced visible macroscopic lung metastasis in comparison to control; however, the combination treatments did not exhibit increased efficacy over single treatments. Analyses of fluorescent microscopic lesions in lungs and lymph nodes showed that all treatment groups significantly reduced these lesions in comparison to control, and that the combination group of α-TEA + celecoxib (1250) significantly reduced these micrometastatic lung lesions better than either

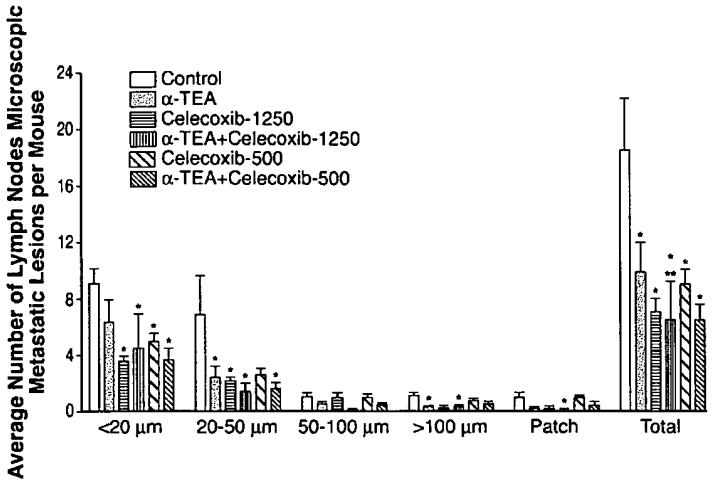

Figure 3. α-TEA and celecoxib reduced lymph node metastasis when administered separately or together. Axillary and brachial lymph nodes from control and treatment groups were flattened and one side examined for fluorescent microscopic lesions. Lesions were placed into five size groupings: <20 μm, 20–50 μm, 50–100 μm, and "patches" containing 100–200 cells. Control (▭), α-TEA (▤), celecoxib (1250 mg/kg diet) (▤), α-TEA + celecoxib (1250 mg/kg diet) (▥), celecoxib (500 mg/kg diet) (▨), and α-TEA + celecoxib (500 mg/kg diet) (▨). Data are depicted as mean ± SE. (*) = significantly different from control, (**) = significantly different from α-TEA.

single treatment. In contrast, the combination treatment of α-TEA + celecoxib (1250) significantly reduced lymph node micrometastasis in comparison to α-TEA alone but not celecoxib (1250) alone. In summary, the combination of α-TEA + celeccoxib (1250) was superior to either single treatment alone in reduction of tumor burden and reduction of micrometastatic lesions in the lungs.

Analyses addressing possible mechanisms of action showed that all treatments, with the exception of celecoxib 500, were effective in inducing apoptosis and inhibiting proliferation of the tumor cells; however, the combination of α-TEA + celecoxib (1250) did not show any advantage over the single treatments. Furthermore, all treatments produced a reduction in blood vessel density; however, only the α-TEA treatment alone and the α-TEA + celecoxib (1250) treatment group were significantly different from control. In this measure of antitumor activity, the combination treatment did not exhibit any advantage over α-TEA treatment alone.

This is the first report on the anticancer properties of α-TEA in a human breast cancer xenograft model; in this model, α-TEA reduced tumor burden and metastasis in a manner similar to studies of α-TEA using a mouse mammary cancer syngeneic animal model [2]. Likewise, this is the first report of the combination effects of α-TEA + celecoxib. Celecoxib is showing promise as a chemopreventive and chemotherapeutic agent for breast cancer. A literature search revealed nine publications showing celecoxib to exhibit chemopreventive and chemotherapeutic properties in rodent models of mammary cancer [4–12]. Clinical trials involving celecoxib and anti-aromatase therapy in hormone-sensitive postmenopausal human breast cancers are in progress [25].

The therapeutic effects of celecoxib have been reported in a human breast cancer xenograft model [7]. Although quite different in design and purpose from our studies, the studies by Blumenthal and co-workers showed that intraperitoneal injections of nontoxic levels of celecoxib at different times during the day was effective in reducing the growth of human MCF-7 breast cancer cells transplanted into nude mice [7]. Although COX-2 message was detected in the MCF-7 cells, COX-2 protein was not detected [7].

Other than the metastatic studies reported here, we are unaware of metastatic studies with celecoxib in a xenograft model. However, the ability of celecoxib to inhibit mammary lung metastasis has been reported in a chemoprevention Her-2/Neu transgenic mouse model [11]. In this model, celecoxib administered by diet at 900 ppm over a period

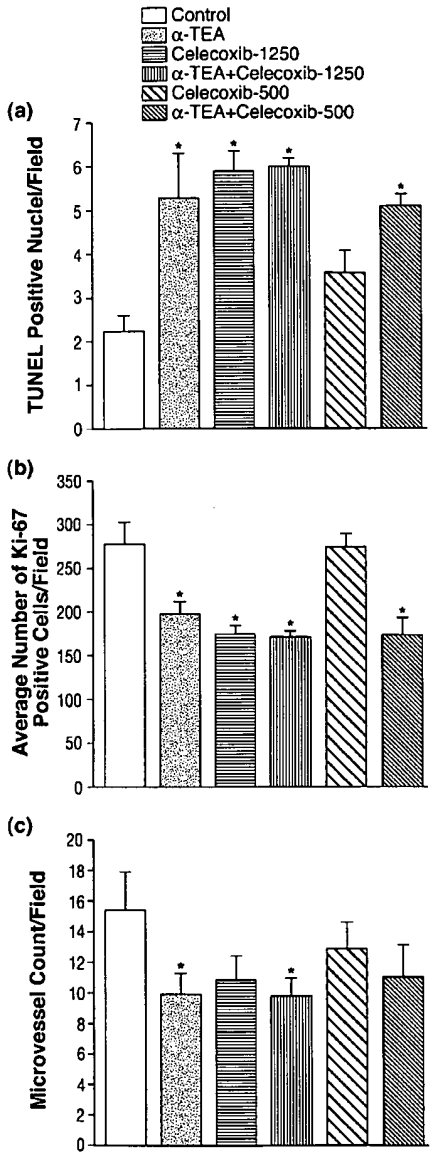

Figure 4. α-TEA and celecoxib increased apoptosis, reduced cell proliferation and reduced blood vessel density. Five micron tumor tissue sections from control and treatment groups (five animals each) were examined immunohistologically for levels of apoptosis by TUNEL (a), cell proliferation by Ki-67 (b), and blood vessel density by CD31 (c). Control (▭), α-TEA (▨), celecoxib (1250 mg/kg diet) (▤), α-TEA + celecoxib (1250 mg/kg diet) (▥), celecoxib (500 mg/kg diet) (▧), and α-TEA + celecoxib (500 mg/kg diet) (▩). (*) = significantly different from control.

of 14 months caused a delay in tumor development, lower tumor incidence, fewer tumors, and reduced incidence of lung metastasis (39% lung metastasis versus 69% for control) [11]. α-TEA has been shown to reduce lung metastasis in a BALB/c 66cl-4-GFP syngeneic mammary cancer model [2].

Since food consumption and weight gain may affect tumor growth, both parameters were evaluated throughout the studies. Food consumption in the α-TEA as well as celecoxib administered separately or in combination groups were not significant different from control. Control and all treatment groups gained weight throughout the studies and there were no significant weight gain differences among control and treatment groups.

Finding that celecoxib administered at 1250 mg/kg/diet was more effective in reducing tumor burden and metastasis than celecoxib at 500 mg/kg/diet was expected since studies in a chemoprevention rat model showed that the ability of celecoxib at levels ranging from 250 to 1500 mg/kg/diet inhibited carcinogen-induced mammary tumor incidence, tumor multiplicity, and tumor volume was dose dependent [9].

The mechanisms whereby α-TEA + celecoxib (1250) are inhibiting tumor growth and metastasis significantly different from either compound administered separately are not known. Although the co-treatments with α-TEA + celecoxib (1250) significantly enhanced apoptosis, reduced cell proliferation, and reduced blood vessel density in tumor tissue, the differences were not significant when compared to the two compounds administered separately. The inability of celecoxib (500 or 1250) alone to significantly reduce blood vessel density in comparison to control was somewhat surprising since data show celecoxib to inhibit COX-2 mediated prostanoid metabolite formation (PGE_2), inhibit tumor growth, and inhibit microvessel density [11, 16, 19]. MDA-MB-435-FL-GFP cells express detectable levels of COX-1 and-2 proteins, and it is well established that celecoxib inhibits COX activity, and it can be inferred that the beneficial effects of celecoxib may be through the inhibition of prostaglandin biosynthesis [9]. A growing body of literature indicates that both COX-2 dependent and COX-2 independent pathways are involved [4, 26–29].

α-TEA induced apoptosis of MDA-MB-435 breast cancer cells in culture is mainly through the JNK signaling pathway [3]. On-going mechanistic signaling pathway studies show that celecoxib most likely does not induce apoptosis *via* MAP kinases. Although preliminary, c-DNA microarray

analyses have identified several genes for further study that are either up-regulated or down-regulated when MDA-MB-435-FL-GFP cells are treated for 24 h with α-TEA and celexoxib, separately and together.

In summary, α-TEA and celecoxib reduced human breast cancer tumor burden and metastasis. Combinations of α-TEA + celecoxib (1250) reduced tumor burden and lung metastasis better than either compound administered separately, providing the rationale for further studies using chemopreventive and chemotherapic animal models.

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ORIGINAL ARTICLE

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Novel vitamin E analogue and 9-nitro-camptothecin administered as liposome aerosols decrease syngeneic mouse mammary tumor burden and inhibit metastasis

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Abstract *Purpose*: To test the anticancer properties of a nonhydrolyzable ether-linked acetic acid analogue of vitamin E, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA), and a derivative of camptothecin, 9-nitrocamptothecin (9-NC) singly and in combination against mouse mammary tumor cells (line 66 clone 4 stably transfected with green fluorescent protein; 66cl-4-GFP) cultured in vitro or transplanted subcutaneously into the inguinal region of female BALB/c mice to form established tumors. *Methods*: Following in vitro treatment of 66cl-4-GFP cells with α-TEA and suboptimal concentrations of 9-NC, singly or in combination, apoptosis was measured by morphological evaluation of nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI), and DNA synthesis arrest was measured by tritiated thymidine uptake. For in vivo analyses α-TEA and 9-NC, both water-insoluble compounds, were formulated into liposomes using dilauroylphosphatidylcholine and administered by aerosol to deliver doses calculated to be 36 and 0.4 μg/mouse per day, respectively, (singly or each separately for combined treatments) 7 days per week. *Results*: Treatment of 66cl-4-GFP cells in culture for 3 days with a combination of α-TEA (10 μg/ml; singly produces 38% apoptosis), and suboptimal concentrations of 9-NC (15.6, 31.3, 62.5, or 125 ng/ml; singly produce 2–7% apoptosis), produced 47%, 58%, 64%, and 69% apoptosis. Likewise, combinations of α-TEA + 9-NC inhibited DNA synthesis more than either agent administered singly. A significant reduction ($P < 0.001$) in growth of subcutaneous transplanted tumors was observed with liposome-formulated and aerosolized delivery of α-TEA + 9-NC to BALB/c mice. The incidence of macroscopic lung metastasis was 83% in control vs 8% in α-TEA-, 9-NC-, or combination-treated mice. Fluorescence microscopic examination of lungs and axillary and brachial lymph nodes showed a statistically significant decrease in metastasis observed in α-TEA-, 9-NC-, and combination- vs control-treated animals. Analyses of primary tumor tissue for proliferation and apoptosis showed treatment groups to have lower Ki-67 and higher terminal deoxynucleotidyl transferase-mediated nick end labeling, respectively. Treatments showed no measurable effects on two angiogenesis parameters, namely intratumoral blood volume as assessed by

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hemoglobin content and intratumoral blood vessel density as assessed with CD31 staining. *Conclusions*: Combination treatments enhanced antiproliferative and proapoptotic activities in cell culture, and when formulated in liposomes and delivered via aerosolization to treat an aggressive and metastatic syngeneic murine mammary tumor, the combination treatment showed a significant reduction in tumor volume in comparison to either treatment alone. Mechanistically, it appears that neither enhanced apoptosis, reduced cell proliferation, nor reduced blood vessel density can fully account for the enhanced effects of the combination treatment.

Keywords Vitamin E analogue α-TEA · 9-nitrocamptothecin · Metastasis · Antitumor · Syngeneic mouse mammary cancer model

Abbreviations α-TEA: 2,5,7,8-Tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid · 9-NC: 9-Nitro-camptothecin · 66cl-4-GFP: BALB/c mouse mammary tumor line 66 clone 4 stably transfected with GFP · DAPI: 4′,6-Diamidino-2-phenylindole · DMSO: Dimethylsulfoxide · GFP: Green fluorescent protein · PECAM-1: Platelet-endothelial cell adhesion molecule · THF: Tetrahydrofuran · TUNEL: Terminal deoxynucleotidyl transferase-mediated nick end labeling

Introduction

Camptothecin, a naturally occurring anticancer agent, was first extracted from the Chinese tree *Camptotheca acuminata* in the early 1960s [19]. Since this time, several less-toxic, clinically active, derivatives have been identified including CPT (irinotecan or CPT-11), 9-amino-camptothecin (9-AC) and 9-nitrocamptothecin (9-NC, or rubitecan) [19]. Camptothecin derivatives are used clinically for the treatment of several forms of cancer including breast, ovarian, lung, and colorectal [7].

Liposomal formulations of 9-NC delivered by aerosol are being investigated as a more effective, less-toxic formulation and delivery method [8, 10, 13, 21]. Dosages used in these studies were chosen based on previous studies in which a range of doses were investigated for both α-TEA [14] and 9-NC [10]. More specifically, efforts to limit 9-NC toxicity by administering it in liposome aerosol at concentrations lower than those traditionally used in mice treated with 9-NC by other routes, have demonstrated this approach to be very effective against human breast, colon and lung cancer xenografts in nude mice [10] and experimental pulmonary metastasis in mice [13]. Furthermore, liposomal formulations of 9-NC delivered by aerosol have been used successful in toxicology studies in dogs [8] and a dose escalation and toxicologic evaluation of cancer patients [21]. A therapeutic trial in cancer patients is in progress (V. Knight, B. Gilbert, and coworkers).

Alpha-tocopherol ether analogue (α-TEA), is a stable, nonhydrolyzable acetic acid derivative of vitamin E (RRR-α-tocopherol) and has been shown recently to be a potent dose-dependent antitumor agent in vitro and in vivo [14]. Because α-TEA is a lipid which complicates i.v. administration and because there is a specific RRR-α-tocopherol transfer protein in the liver that selectively mediates the transfer of RRR-α-tocopherol into lipoproteins thus limiting bioavailability following oral administration of vitamin E compounds other than RRR-α-tocopherol, we are investigating the formulation of α-TEA into liposomes and aerosol delivery as a potential clinically-relevant cancer treatment strategy. In preclinical syngeneic transplantable mouse mammary cancer studies, α-TEA formulated into liposomes and delivered by aerosol has been shown to: (1) significantly decrease tumor growth rate ($P < 0.001$) over 17 days of treatment; (2) completely inhibit visual macroscopic metastasis, reducing incidence from 40% in control animals to 0% in animals treated with α-TEA; and (3) significantly reduce microscopic metastasis ($P < 0.002$) [14]. In all in vivo studies conducted to date, mice receiving α-TEA have exhibited no significant difference in mean body weight or any visible indication of toxicity such as lethargy or loss of hair [14]. Thus, based on its anticancer effectiveness in vivo and lack of overt toxicity, α-TEA is a promising agent for investigation separately and in combination with other anticancer drugs.

Although the antitumor mechanisms of action of 9-NC and α-TEA are not completely understood, 9-NC has been shown to inhibit cell growth by inhibition of DNA synthesis and induction of cell death by apoptosis [2, 4, 17] and α-TEA has been shown to be a potent proapoptotic agent [14]. Of potential interest to these studies are recent investigations suggesting that the death receptor CD95 (APO-1/Fas) signaling pathway is important for 9-NC- and α-TEA-induced apoptosis [4, 21]. Chatterjee et al. [4] have shown that 9-NC induces de novo synthesis of both CD95 ligand and CD95 as well as downregulation of the antiapoptotic protein c-FLIP (Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein) in human prostate carcinoma DU145 cells.

Taken together with the findings of Shun et al. [18] showing that functional knockdown of the Fas signaling pathway by treatment of human MDA-MB-431 breast cancer cells with neutralizing antibodies to CD95 partially abrogates α-TEA-induced apoptosis, we speculated that a combination of α-TEA and 9-NC might exhibit additive or synergistic proapoptotic activity. Of additional interest in considering potential cooperative activity between α-TEA and 9-NC is experimental evidence demonstrating that both of these agents exhibit antimetastatic potential in animal models although mechanistic insights into this aspect of their antitumor activities are currently lacking [13, 14]. α-TEA has been shown in our laboratory to be effective in reducing tumor burden and inhibiting metastasis, but it does not totally eliminate tumor growth or metastasis [14].

The rationale for the animal studies reported here were based on promising cell culture data presented in this paper showing that combinations of α-TEA + 9-NC enhanced apoptosis more than either agent administered separately. Thus, studies were initiated to investigate the hypothesis that α-TEA in combination with 9-NC would enhance the in vivo therapeutic effects over either drug administered separately. The primary outcomes from these studies were the ability of α-TEA and 9-NC in combination to reduce tumor burden and inhibit metastasis to a greater degree than either compound administered separately.

We report here that the combination of α-TEA + 9-NC produced a higher degree of apoptosis and DNA synthesis arrest in culture than either agent alone, significantly reduced subcutaneous syngeneic mouse mammary tumor growth ($P < 0.001$) to a greater degree than either agent alone, reduced the total incidence of visible macroscopic metastasis by 90%, reduced the total incidence of microscopic lung metastasis by 71%, and axillary and brachial lymph node metastasis by 88%, reduced the number of proliferating tumor cells in vivo by 68%, and increased tumor cells undergoing apoptosis by 90%. Investigations of parameters of tumor angiogenesis revealed that neither reductions in primary tumor blood volume nor number of CD31 positive-staining blood vessels in tumors appear to be involved in the antitumor effects of α-TEA and 9-NC administered separately or in combination.

Materials and methods

α-TEA and 9-NC and in vitro treatments

α-TEA was synthesized, its structure confirmed, and production scaled up to provide sufficient amounts for preclinical animal studies by one of the authors (J.A.) as previously described [14]. 9-NC was purchased from ChemWerth (Woodbridge, Ct.). For in vitro treatments, stock (40 m *M* and 318 μ*M*) solutions of α-TEA and 9-NC were made in ethanol (EtOH) and dimethyl sulfoxide (DMSO), respectively. Final concentrations of chemicals were achieved by dilution in medium. Final concentrations (v/v) of EtOH and DMSO in the cell treatments were 0.1% and 0.1%, respectively.

66cl-4-GFP murine mammary tumor cell line

The 66cl-4 cell line is a mouse mammary tumor cell line originally derived from a spontaneous mammary tumor in a Balb/cfC3H mouse and later isolated as a 6-thioguanine-resistant clone [6, 15]. The 66cl-4 cells were stably transfected with the enhanced green fluorescent protein and selected for a high degree of fluorescence by L.-Z.S. (one of the authors). 66cl-4-GFP cells are highly metastatic with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing fluorescent microscopic metastases in the lungs 26 days after subcutaneous injection of 2×10^5 tumor cells into the inguinal area [14]. 66cl-4-GFP cells were maintained as monolayer cultures in McCoy's medium (Invitrogen Life Technologies, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, Calif.), 100 μg/ml streptomycin, 100 IU/ml penicillin, 1× (v/v) nonessential amino acids, 1× (v/v) MEM vitamins, 1.5 m *M* sodium pyruvate, and 50 μg/ml gentamicin (Invitrogen Life Technologies). Treatments were given using this same McCoy's supplemented medium except the fetal bovine serum content was reduced to 5%. Cultures were routinely examined to verify absence of *Mycoplasma* contamination.

Determination of DNA synthesis by incorporation of [^{3}H]thymidine

The effects of α-TEA and 9-NC, separately and in combination, on inhibition of DNA synthesis of 66cl-4-GFP cells were determined by [^{3}H]thymidine incorporation as described previously [3]. Briefly, 66cl-4-GFP cells at 2×10^4 cells/well in 96-well plates were cultured separately and in combination with 10 μg/ml of α-TEA and 8, 15, 31, or 62 ng/ml of 9-NC for 24 h, adding 0.5 μCi [^{3}H]thymidine 6 h prior to harvesting the cells. [^{3}H]Thymidine incorporation was measured using a Beckman LS5000TD liquid scintillation counter.

Determination of apoptosis by morphological evaluation of DAPI-stained nuclei

Apoptosis was determined using previously published procedures [14, 18]. Cells in which the nucleus contained condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Data are reported as percentage of apoptotic cells/cell population (i.e., number apoptotic cells/total number of cells counted). Three different microscopic fields were examined, and 200 cells counted at each location for a minimum of 600 cells counted per slide. Apoptotic data are presented as the mean ± SD for three independently conducted experiments.

BALB/c mice

Female BALB/c mice at 6 weeks of age (20–25 g in weight) were purchased from Jackson Laboratories (Bar Harbor, Me.). Mice, five per cage, were housed at the Animal Resource Center at the University of Texas at Austin at 74 ± 2°F with 30–70% humidity and a 12-h alternating light/dark cycle. Mice were given water and standard laboratory chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison, Wis.) ad libitum. Guidelines for the humane treatment of animals were followed as approved by

the University of Texas Institutional Animal Care and Use Committee.

Tumor cell inoculation and tumor growth measurement

Mice were injected (100 µl) subcutaneously with 2×10^5 cells in the inguinal area at a point equidistant from the fourth and fifth nipples on the right side. A total of 48 mice (12 mice/group) were assigned to α-TEA, 9-NC, α-TEA + 9-NC, or liposome-only (control group) such that the mean tumor volume of each group was closely matched. The groups had average tumor volumes per group of 1.55, 1.34, 1.26, and 1.60 mm^3, respectively, at the start of the treatments, which were begun 9 days after tumor cell inoculation. Tumors were measured using calipers every other day, and volumes were calculated using the formula: volume (mm^3) = [width (mm^2)×length (mm)]/2 [5]. Body weights were determined weekly.

Preparation of α-TEA and 9-NC liposomes

α-TEA/liposome and 9-NC/liposome were prepared as previously described [10, 14]. Briefly, the lipid (1,2-dilauroyl-*sn*-glycerol-3-phosphocholine (DLPC); Avanti Polar-Lipids, Alabaster, Ala.) at a concentration of 120 mg/ml was dissolved in *t*-butanol (Fisher Scientific, Houston, Tx.) then sonicated to obtain a clear solution. α-TEA at 40 mg/ml was dissolved in *t*-butanol and vortexed until all solids were dissolved. The two solutions were then combined in equal amounts (v/v) to achieve the desired ratio of 1:3 α-TEA/liposome, mixed by vortexing, frozen at −80°C for 1–2 h, and lyophilized overnight to a dry powder before storing at −80°C until needed. Each treatment vial contained 75 mg of α-TEA. 9-NC treatments were made as described previously [10]. Briefly, 9-NC (100 mg/ml) was dissolved in DMSO and warmed to 37°C. DLPC (100 mg/ml) was dissolved in *t*-butanol. Drug and phospholipid solutions were added at a 1:50 (w/w) ratio, and mixed by vortexing. Samples were frozen at −80°C for 1–2 h before lyophilizing overnight. Each treatment vial contained 1 mg of 9-NC.

Aerosol delivery

All treatments were administered by aerosol as described previously [12, 14]. Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, Pa.) producing a 10 l/min airflow was used with an AeroTech II nebulizer (CIS-US, Bedford, Mass.) to generate aerosol. Particle sizes of liposomal formulated α-TEA and 9-NC aerosol discharged from the AeroTech II nebulizer were determined using an Anderson Cascade Impactor to have a mass median aerodynamic diameter (MMAD) of 2.01 µm ± geometric SD of 2.04 and 1.6 µm ± geometric SD of 2.04, respectively [10, 14].

Before nebulization, the α-TEA/lipid powder (75 mg/vial) and 9-NC/lipid powder (1 mg/vial) were brought to room temperature then reconstituted by adding 3.75 and 5 ml distilled water, respectively, to achieve the final desired concentration of 20 mg/ml α-TEA and 200 µg/ml 9-NC. The mixtures were allowed to swell at room temperature for 30 min with periodic inversion and vortexing, and then all 3.75 ml of α-TEA or 5 ml of the 9-NC were added to the nebulizer. 9-NC was administered 8 h after administration of α-TEA. Treatments were administered 7 days per week. Animals were exposed to aerosol until all α-TEA or 9-NC was aerosolized (approximately 15 min). Based on previous calculations of amount of aerosolized α-TEA or 9-NC delivered to mice under these treatment conditions, it was estimated that approximately 36 µg α-TEA and 0.4 µg 9-NC per mouse per day were deposited in the lungs, respectively. Calculations were based on a 25-g mouse with an inhalation volume of 1 l/min/kg body weight and an average aerosol retention factor of 30% [12].

Lung and lymph node metastasis

Macroscopic metastases in all five lung lobes were counted visually at the time the mice were killed. Fluorescent microscopic metastases were counted using a Nikon fluorescence microscope (TE-200; ×200 magnification) as described previously [14]. For analyses, the left lung lobe was flattened and the top and bottom surfaces were scored for fluorescent green microscopic metastases. Fluorescent microscopic metastases were scored by size into three size groupings: < 20, 20–50, and > 50 µm. On the basis of a typical 66cl-4-GFP tumor cell size of 10–20 µm in diameter, the < 20 µm grouping was thought to represent solitary cells, the 20–50 µm grouping two to five cells, and the > 50 µm grouping microscopic metastases of more than two to five cells.

Ki-67 staining for detection of proliferation in tumor tissue

Deparaffinized sections (5 µm) of tumor tissue were used to assess proliferation using antibody to the Ki-67 antigen, a nuclear antigen expressed in cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked using a 3% H_2O_2 solution for 10 min followed by washing with PBS. Rabbit serum (10%) in PBS was applied to 5 µm tumor tissue sections to block nonspecific antibody binding, before incubation with primary antibody (rat-anti-mouse Ki-67 antibody,1:200 dilution; DAKO Corporation, Carpinteria, Calif.) overnight at 4°C. After primary antibody incubation, slides were washed then incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, Calif.) at a 1:200 dilution for 30 min at room temperature. Sections were then incubated with avidin–biotin complex (ABC-HRP; Vector Laborato-

ries) for 30 min at room temperature. Immunoreactivity was visualized via incubation with diaminobenzidine dihydrochloride. Slides were lightly counterstained with hematoxylin. Ki-67-positive stained cells were counted in five separate fields per sample. The data are presented as the means ± SE of all tumors in each group ($n = 11$ for α-TEA, 9-NC, and combination groups; $n = 12$ for control group).

TUNEL assay for detection of apoptosis in tumor tissue

Deparaffinized sections (5 μm) of tumor tissue were used to assess apoptosis using reagents supplied in the ApopTag in situ apoptosis detection kit (Intergen, Purchase, N.Y.) according to the manufacturer's instructions. Nuclei that stained brown were scored as positive for apoptosis and those that stained blue were scored as negative. At least 16 separate random fields at high power (×400 magnification) were scored per tumor. The data are presented as the mean ± SE numbers of apoptotic cells counted in seven or more individual tumors from each treatment group.

CD31 staining for determination of blood vessel formation in tumor tissue

Immunohistochemistry was used to assess the presence of the endothelial antigen CD31 (also referred to as PECAM-1, platelet-endothelial cell adhesion molecule) as an indicator of small capillaries in primary tumor tissue. Formalin-fixed paraffin-embedded tissues were sectioned (5 μm) and tissue sections adhered onto charged slides (Fisher Scientific, Chicago, Ill.). Tissue sections were deparaffinized, hydrated through a series of graded ethanol solutions to water and endogenous peroxidase activity blocked by incubation in 3% H_2O_2 for 10 min. Tissue sections were then pretreated with 0.06% Protease Type XXIV (Sigma) for 10 min at room temperature before incubation with antibody to CD31 (PECAM-1; PharMingen, San Diego, Calif.) at a 1:400 dilution overnight at 4°C. Detection utilized the Tyramide Signal Amplification Biotin System-Peroxidase (PerkinElmer Life Sciences, Boston, Mass.) with diaminobenzidine dihydrochloride development. For contrast, the sections were lightly counterstained with hematoxylin. Entire tumor sections ($n = 3$ for α-TEA and 9-NC groups; $n = 4$ for combination and control groups) were scored for CD31-stained vessels and then were adjusted for tumor size, by dividing number of CD31 vessels by length×width of tumor tissue mounted on the slide.

Hemoglobin assay

Frozen tumor samples ($n = 4$ for each treatment group) were analyzed to determine hemoglobin levels in an effort to corroborate the blood vessel density data obtained by CD31 staining. Tumors were carefully cleaned to remove any surface blood vessels and then hemoglobin was extracted as described previously [1]. Briefly, frozen samples were weighed, frozen in liquid nitrogen and pulverized using mortar and pestle. Extraction was performed with ice-cold buffer containing 10 m *M* Tris (pH 7.5), 0.1 *M* NaCl, 0.5% Triton-X-100, 1.0 m *M* ethylene diaminetetraacetic acid, and 0.1 m *M* phenylmethylsulfonyl fluoride. Six milliliters of extraction buffer was used for each gram of tissue. Extracts were centrifuged at 10,000 *g* for 15 min, and hemoglobin was measured via a hemoglobin extraction kit (Sigma) following the manufacturer's instructions. The hemoglobin content in systemic blood obtained at the time the animals were killed was also measured to obtain the hemoglobin level per unit of blood so that the blood volume of each tumor could be calculated.

Statistical analyses

Differences among the treatment groups for the cell culture data were evaluated using the unpaired *t*-test with unequal variances. The degree of enhancement of apoptosis among cells cotreated with α-TEA + 9-NC was evaluated using two-way analysis of variance. Animal numbers for experiments were determined by power calculations derived from data generated in previous studies. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance with Tukey post-test using SPSS (SPSS, Chicago, Ill.). The significance of the differences in number of fluorescent microscopic and macroscopic metastases per group, Ki-67-stained cells per group, and TUNEL-positive nuclei per group were determined using the two-tailed Mann-Whitney rank test using Prism software version 3.0 (GraphPad, San Diego, Calif.). A level of $P < 0.05$ was regarded as statistically significant.

Results

Structure of 9-NC and α-TEA

The structures of 9-NC and α-TEA are depicted in Fig. 1. 9-NC is a less toxic, clinically active derivative of camptothecin, a pentacyclic alkaloid isolated from the Chinese tree *C. acuminata* [22]. α-TEA is a nonhydrolyzable ether analogue of natural vitamin E, RRR-α-tocopherol. α-TEA differs from RRR-α-tocopherol by an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage [14].

α-TEA, 9-NC, and combination treatments induce apoptosis in 66cl-4-GFP cells in cell culture

66cl-4-GFP cells treated with α-TEA (5 μg/ml) for 3 days exhibited 8% apoptosis (Fig. 2a). Cells treated

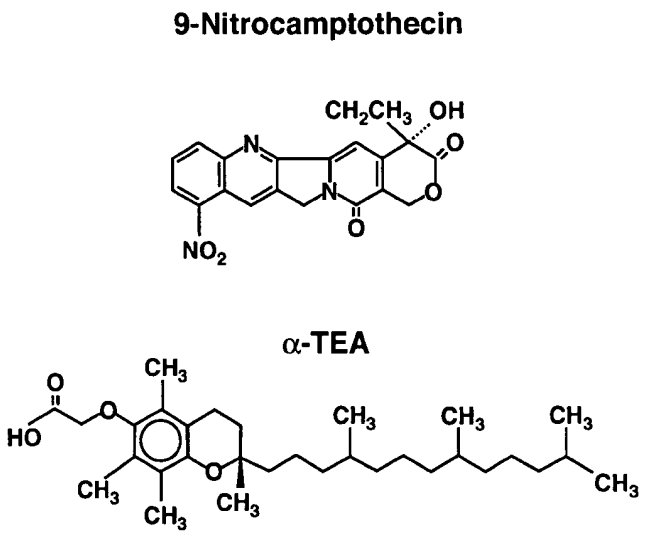

Fig. 1 Structure of 9-NC and α-TEA

with 9-NC (15, 31, 62, or 125 ng/ml) exhibited 2%, 4%, 6% and 7% apoptosis, whereas cells treated with combinations of 5 μg/ml α-TEA plus various concentrations of 9-NC exhibited elevated levels of apoptosis (14%, 24%, 34% and 61%). A significant increase in apoptosis was observed for two of the cotreatments (5 μg/ml α-TEA plus 62 or 125 ng/ml 9-NC) in comparison to α-TEA and 9-NC alone ($P < 0.045$, 0.00072; and $P < 0.04$, 0.0028, respectively; Fig. 2a).

66cl-4-GFP cells treated with 10 μg/ml α-TEA for 3 days exhibited 38% apoptosis, and treatment with 9-NC at the four different doses induced 2%, 4%, 6% and 7% apoptosis, whereas cells treated with combinations of 10 μg/ml α-TEA plus various concentrations of 9-NC exhibited elevated levels of apoptosis (47%, 58%, 64% and 68%; Fig. 2b). A significant increase in apoptosis was observed for three of the cotreatments (10 μg/ml α-TEA plus 31, 62, and 125 ng/ml 9-NC) in comparison to

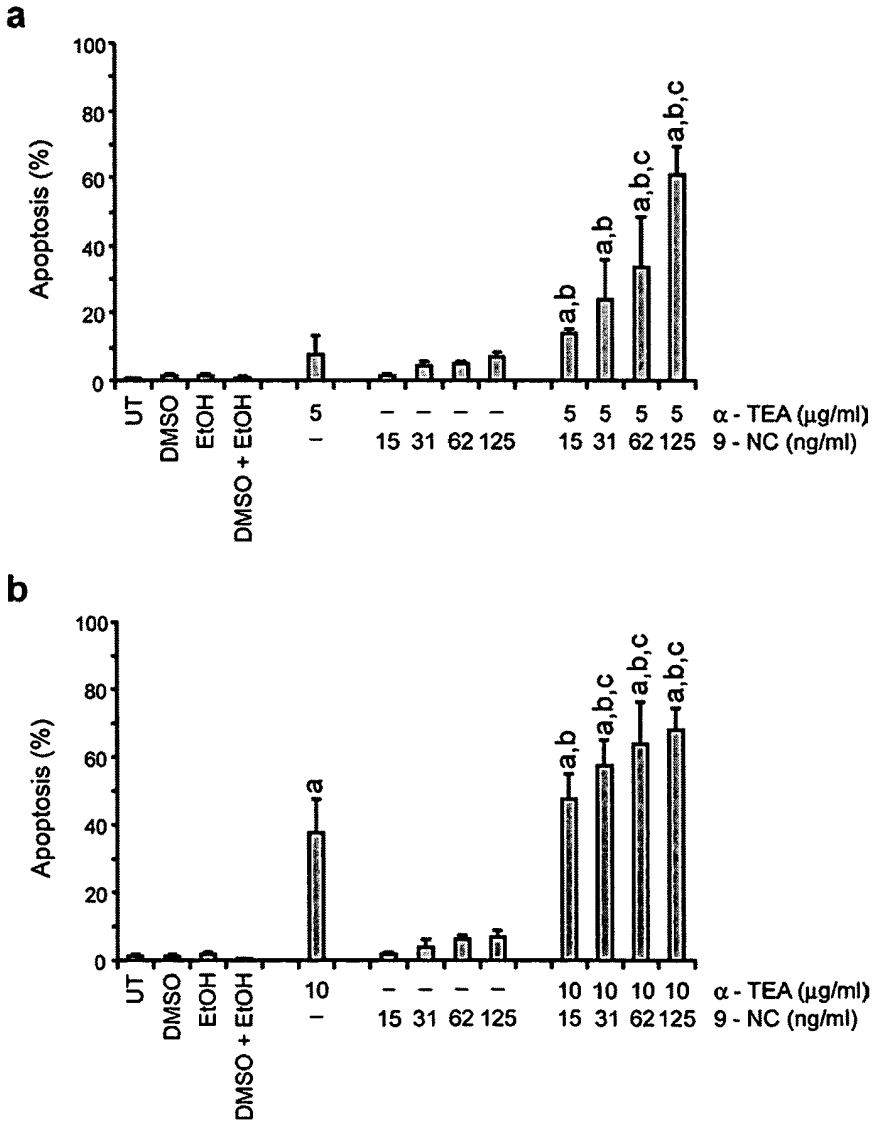

Fig. 2a, b α-TEA and 9-NC singly and in combination induced 66cl-4-GFP murine mammary cells to undergo apoptosis. Cells were treated with α-TEA at (**a**) 5 μl/ml or (**b**) 10 μg/ml and with various levels of 9-NC for 3 days. Nuclei were labeled with the fluorescent DNA-binding dye DAPI. Nuclei exhibiting condensed chromatin or fragmented DNA were scored as apoptotic. Data are means ± SD of three separate experiments ([a]significantly different from control, [b]significantly different from 9-NC, [c]significantly different from α-TEA; $P < 0.05$)

Table 1 Inhibition of DNA synthesis in 66cl-4-GFP mammary cancer cells following treatment with α-TEA, 9-NC, and their combinations. 66cl-4-GFP cells cultured at 2.0×10^4 cells/well in 96 well-plates were treated with α-TEA (10 μg/ml), 9-NC (8, 15, 31, or 62 ng/ml), combinations of α-TEA (10 μg/ml) + 9-NC (8, 15, 31, or 62 ng/ml) or DMSO + EtOH (control), or untreated, for 1 day. DNA synthesis was monitored by [^{3}H]thymidine uptake. DNA synthesis arrest was calculated by comparing [^{3}H]thymidine uptake by cells receiving treatments with [^{3}H]thymidine uptake by cells receiving no treatment. Values are means ± SD of three separate experiments

Treatment	DNA synthesis arrest (%)	Fold increase in inhibition in comparison to cells treated with	
		α-TEA alone	9-NC alone
α-TEA (10 μg/ml)	35 ± 5.0		
9-NC (8 ng/ml)	29 ± 5.5		
9-NC (15 ng/ml)	46 ± 4.9		
9-NC (31 ng/ml)	77 ± 4.4		
9-NC (62 ng/ml)	90 ± 1.5		
α-TEA (10 μg/ml) + 9-NC (8 ng/ml)	53 ± 2.5	1.5	1.8
α-TEA (10 μg/ml) + 9-NC (15 ng/ml)	65 ± 2.0	1.9	1.4
α-TEA (10 μg/ml) + 9-NC (31 ng/ml)	84 ± 2.5	2.4	1.1
α-TEA (10 μg/ml) + 9-NC (62 ng/ml)	92 ± 1.0	2.6	1.0
DMSO + EtOH	6 ± 3.6		

α-TEA and 9-NC alone ($P < 0.050$, 0.034 and 0.019; and $P < 0.0008$, 0.001 and 0.0007, respectively; Fig. 2b).

Untreated, EtOH, DMSO, and EtOH + DMSO controls exhibited background levels of apoptosis of 1%, 1%, 2%, and < 1% apoptosis, respectively (Fig. 2).

α-TEA, 9-NC, and combination treatments inhibit DNA synthesis in 66cl-4-GFP cells in cell culture

66cl-4-GFP cells were treated to determine the effect of each agent separately and in combination on DNA synthesis (Table 1). Cells treated with 10 μg/ml α-TEA exhibited 35% reduction in DNA synthesis when compared to untreated controls. 9-NC at 8, 15, 31, or 62 ng/ml inhibited DNA synthesis by 29%, 46%, 77%, and 90%, respectively, in comparison to untreated controls. Cells treated with combinations of 10 μg/ml α-TEA plus the various concentrations of 9-NC exhibited 53%, 65%, 84%, and 92% DNA synthesis inhibition in comparison to untreated controls. EtOH + DMSO vehicle-treated cells exhibited 6% DNA synthesis arrest in comparison to untreated control cells (Table 1).

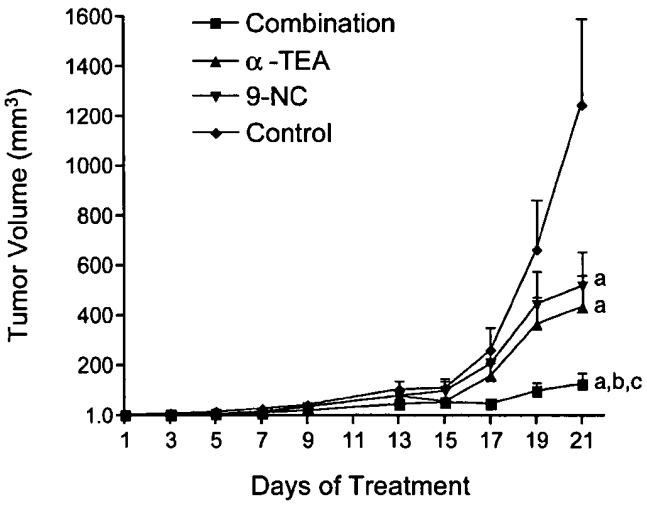

Fig. 3 Aerosolized α-TEA, 9-NC, and combination treatments reduced tumor volume. Treatments were started 9 days after subcutaneous injection of 2×10^5 66cl-4-GFP cells/mouse and continued 7 days per week for 3 weeks ([a]significantly different from control, [b]significantly different from 9-NC, [c]significantly different from α-TEA; $P < 0.05$)

Liposomal formulated α-TEA, 9-NC, and combination treatments delivered by aerosolization decreased 66cl-4-GFP tumor burden in BALB/c mice

Mean primary tumor volumes of aerosol control-treated animals over 21 days of treatment were significantly higher than all other groups ($P < 0.001$; Fig. 3; mean ± SE 1245 ± 344 mm^3). Both 9-NC- and α-TEA-treated groups had mean tumor volumes significantly lower than control animals ($P < 0.001$; means ± SE 518 ± 134 mm^3 and 434 ± 124 mm^3, respectively); and the size of the tumors in the α-TEA and 9-NC single treatment groups were not significantly different from one another ($P < 0.2$; means ± SE 518 ± 134 mm^3 and 434 ± 124 mm^3, respectively). Animals receiving α-TEA + 9-NC had mean tumor volumes over 21 days of treatment that were significantly lower than those of control animals and those of both α-TEA- and 9-NC-treated animals ($P < 0.001$; Fig. 3; mean ± SE 126 ± 42 mm^3). There were no significant differences in mean body weights among any of the treatment or control groups (data not shown).

Liposomal formulated α-TEA, 9-NC, and combination treatments delivered in aerosol suppressed 66cl-4-GFP lung and lymph node metastasis in BALB/c mice

At the time the animals were killed, all five lung lobes and axillary and brachial lymph nodes were taken,

Table 2 66cl-4-GFP mammary cancer cell lung metastasis in BALB/c mice receiving liposomal formulated α-TEA, 9-NC, or α-TEA + 9-NC, or liposome control by aerosol

Treatments delivered by aerosol	No. of animals per group with visible macroscopic lung metastases[a]	Total no. of visible lung macroscopic metastatic foci[b]
Control	10/12	36
9-NC	1/12	2
α-TEA	1/12	2
α-TEA + 9-NC	1/12	2

[a]Macroscopic metastatic lesions in all five lung lobes for each animal in all treatment groups were counted visually at the time the animals were killed.
[b]The values presented the total number of visible lung macroscopic metastases observed in the 12 mice in each group.

examined visually for macroscopic metastatic lesions and frozen for subsequent analyses of microscopic metastases by fluorescent microscopy. Visible macroscopic lung metastases were observed in 1 of 12 animals (8%) in each of the α-TEA, 9-NC, and α-TEA + 9-NC treatment groups ($P < 0.002$), in comparison to 10 of 12 mice (83%) in the aerosol control group (Table 2). Total numbers of macroscopic lung lesions observed differed greatly between the control and treatment groups, with a total of 36 lesions observed in the aerosol control group in comparison to only 2 lesions observed in each of the treatment groups (Table 2). No macroscopic metastases were observed in any of the lymph nodes.

Use of a Nikon fluorescence microscope permitted measurement and classification of green fluorescing microscopic metastases in lung tissue into three size groupings (< 20, 20–50, and > 50 μm; Fig. 4a). Since the tumor cells are approximately 10–20 μm in diameter, the < 20 μm group most likely represents single cells. This analysis showed a decrease in the number of microscopic lung metastases in all three size groups and all three treatment groups in comparison to the control group. The mean numbers of microscopic metastases in the α-TEA treatment group (28.4 ± 3.3, $n = 11$), the 9-NC treatment group (43.5 ± 8.7, $n = 11$), and the combination treatment group (27.6 ± 5.2, $n = 12$) were significantly reduced in comparison to the aerosol control group (96.7 ± 12.2, $n = 12$; $P < 0.0001$, < 0.002, and < 0.0001, respectively). However, the mean numbers of microscopic metastases in the α-TEA + 9-NC combination treatment group and the α-TEA and 9-NC single treatment groups were not significantly different.

The number of microscopic metastases in the axillary and brachial lymph nodes from the different treatment groups were significantly reduced in comparison to the control group (α-TEA, $P < 0.0001$; 9-NC, $P < 0.05$; α-TEA + 9-NC, $P < 0.0004$; Fig. 4b). It is important to note that 71%, 33%, and 52% of lymph nodes from mice treated with α-TEA, 9-NC, or α-TEA + 9-NC, respectively, had no microscopic metastases, whereas

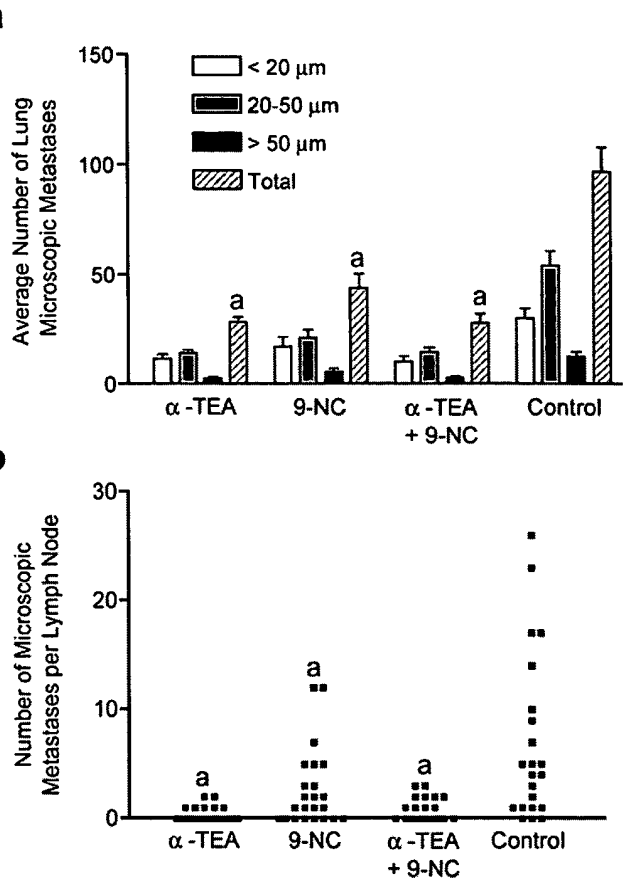

Fig. 4a, b Aerosolized α-TEA, 9-NC, and combination treatments inhibited lung and lymph node metastasis. The number of fluorescent microscopic metastases (**a**) on the surface of the left lung lobe or (**b**) on the surface of individual lymph nodes were determined ([a]significantly different from control; $P < 0.05$)

only 14% of lymph nodes from the control mice were free of microscopic metastases.

Inhibition of cell proliferation by α-TEA, 9-NC, and combination treatments in vivo

Tumor sections from each of the treatment groups as well as the aerosol control group were examined by immunohistochemistry for proliferation status using the nuclear Ki-67 antigen expressed in proliferating cells as a biomarker. The number Ki-67-positive cells in tumors from mice treated with α-TEA was 106 ± 41.4 cells/field (mean ± SE) compared with 241 ± 35.7 cells/field in tumors from aerosol control mice ($P < 0.008$; Fig. 5a). The number Ki-67-positive cells in tumors from mice treated with 9-NC (218 ± 45.6 cells/field, mean ± SE) showed no significant decrease compared with the number in tumors from aerosol control mice (Fig. 5a). The number Ki-67-positive in tumors from mice treated with α-TEA + 9-NC (77 ± 20.2 cells/field, mean ± SE) was also significantly lower than the number in tumors from aerosol

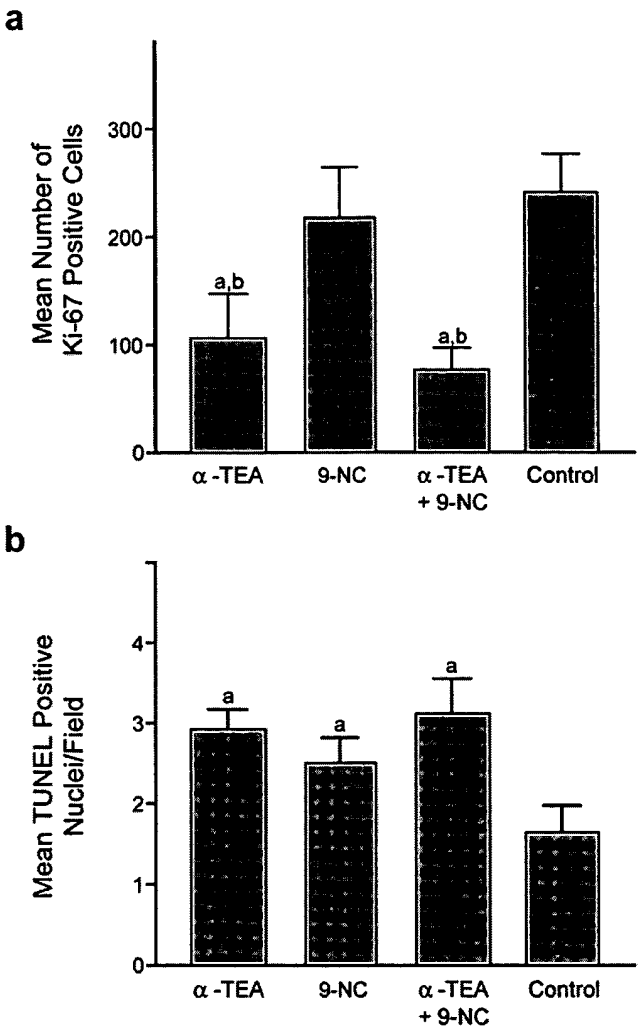

Fig. 5a, b Aerosolized α-TEA, 9-NC, and combination treatments inhibited 66cl-4-GFP cell proliferation and increased apoptosis in vivo. Cell proliferation (**a**) and apoptosis (**b**) were determined using immunohistochemical analyses of 5-μm tumor sections ([a]significantly different from control, [b]significantly different from 9-NC; $P < 0.05$)

control mice ($P < 0.001$; Fig. 5a). The mean numbers of Ki-67-positive cells in tumors from mice treated with α-TEA, and α-TEA + 9-NC were significantly lower than in tumors from mice treated with 9-NC only ($P < 0.02$, $P < 0.004$, respectively). However, there was no significant difference in the mean number of Ki-67 positive cells between the α-TEA treated group and the α-TEA + 9-NC combination treatment group (Fig. 5a).

Induction of apoptosis by α-TEA, 9-NC, and combination treatments in vivo

In view of the in vitro data showing that combinations of α-TEA + 9-NC inhibited 66cl-4-GFP tumor cell growth via induction of apoptosis to a greater extent than either agent administered singly, tumors from each of the treatment groups were examined for apoptosis using TUNEL staining of 5-μm tumor sections. Tumors from mice treated with α-TEA, 9-NC, and α-TEA + 9-NC had a mean ± SE of 2.93 ± 0.24, 2.51 ± 0.31, and 3.12 ± 0.42 apoptotic cells/field, respectively, whereas tumors from aerosol control mice had a mean ± SE of 1.64 ± 0.32 apoptotic cells/field ($P < 0.009$, $P < 0.05$, and $P < 0.014$, respectively; Fig. 5b).

α-TEA, 9-NC, and combination treatments did not have a significant effect on tumor angiogenesis as determined by CD31 staining and hemoglobin content

CD31 expression and hemoglobin levels in tumors were examined in an effort to determine if the antimetastatic effects of α-TEA, 9-NC, and α-TEA + 9-NC treatments might be due to reduced blood vessel formation. Tumors were examined for the presence of blood vessels using staining for CD31 (PECAM-1), an endothelial cell marker (Fig. 6a). There were no significant differences in the number of blood vessels per tumor section among the treatment and control groups when standardized for tumor size (Fig. 6a). As an alternate method for obtaining information on intratumoral blood vessel density, tumor blood volume was determined using a hemoglobin content assay (Fig. 6b). Consistent with the CD31 staining results, tumors tested from treatment groups did not show a reduction in blood volume when compared to aerosol-treated controls.

Discussion

The studies reported here showed that α-TEA, a new vitamin E analogue, and 9-NC significantly increased tumor cell death by apoptosis and blocked cell proliferation in cell culture, and significantly reduced tumor burden in a mouse mammary tumor model.

9-NC is a less-toxic derivative of camptothecin. Preclinical animal data show 9-NC to inhibit human breast cancer xenograft growth in athymic nude mice by as much as 100% [9, 10]. 9-NC, which is currently in clinical trials for treatment of ovarian and pancreatic cancer, is being considered for treatment of breast cancer [11, 16, 20]. Although 9-NC possesses impressive anticancer activity, it also exhibits significant toxic effects, thus the need for seeking treatment strategies that might circumvent this problem by combining 9-NC at a lower dosage with another anticancer drug. In the cell culture studies reported here, combinations of α-TEA (10 μg/ml) and suboptimal doses of 9-NC (31, 62, or 125 ng/ml) gave significantly greater levels of apoptosis than either drug administered as a single agent. Furthermore, combinations of α-TEA + suboptimal doses of 9-NC resulted in higher levels of DNA synthesis arrest than either drug alone. Based on these encouraging in vitro results, investigations of combination treatment on tumor growth and metastasis in a syngeneic transplantable mouse mammary cancer model were undertaken. No

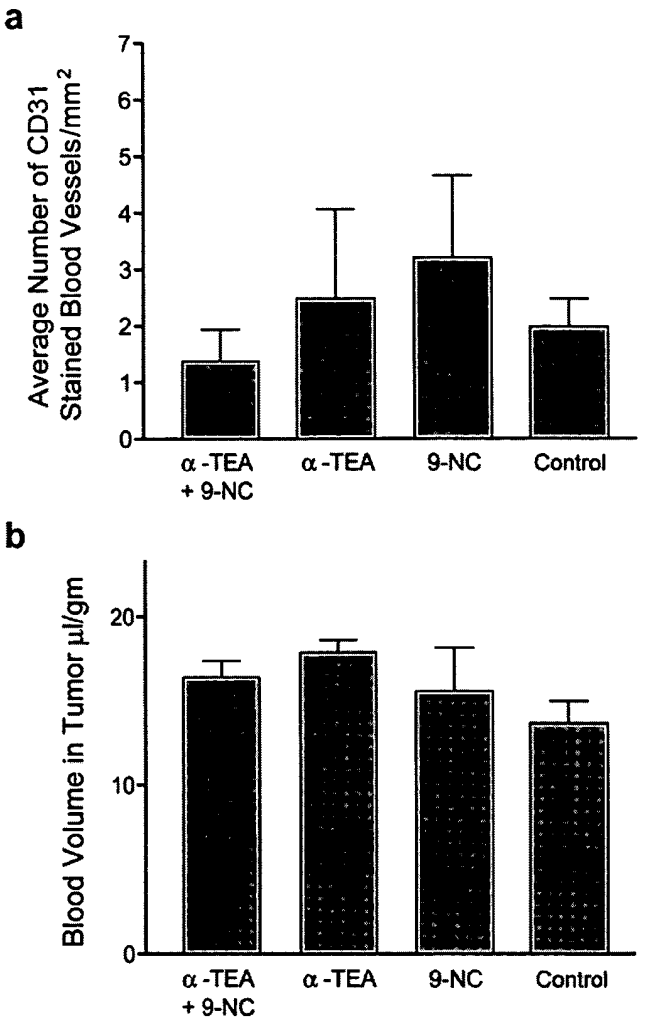

Fig. 6a, b Aerosolized α-TEA, 9-NC, and combination treatments had no effect on the number of blood vessels or blood volume in 66cl-4-GFP tumors. **a** CD31-positive blood vessels per entire 5 µm tumor section were counted then adjusted for tumor size by dividing by length × width of tumor tissue. **b** Levels of hemoglobin in tumor extracts and systemic blood from each mouse were determined using a hemoglobin assay kit, and the corresponding blood volume of each tumor was calculated. Data presented are means ± SE

differences in mean body weights among control or treatment groups were observed (data not shown).

We do not understand the mechanisms whereby a significant increase in apoptosis as measured by TUNEL, a significant decrease in cell proliferation as measured by Ki-67, or a significant decrease in blood vessel density as measured by CD31 or hemoglobin levels was not seen in the combination treatment of 9-NC + α-TEA in comparison to the single treatments since the combination treatment was capable of reducing tumor burden significantly better than the single treatments alone.

Likewise, the mechanisms whereby α-TEA singly and in combination with 9-NC reduced both visible and microscopic metastases are not known. Based on data showing no significant differences between treatment and control groups in CD31 expression and hemoglobin levels in tumors, it appears that neither the single agent treatments nor the combination treatment were effective in reducing tumor volume or preventing metastases by reduction of number of blood vessels or blood volume in tumors.

Data showing that α-TEA, 9-NC, and α-TEA + 9-NC inhibit microscopic metastases in lymph nodes are of interest in that metastasis was reduced in a site that is not directly targeted by the aerosol treatment. It is possible that these drugs are having a direct killing effect on cells at secondary sites. Alternatively, since a high percentage of lymph nodes in the treatment groups did not show any metastasis, it is possible that the treatments are preventing tumor cells from trafficking from the primary subcutaneous tumor to lungs and lymph nodes via the lymphatic system.

In summary, these results suggest that combination treatments using α-TEA, a novel vitamin E analogue, and lower doses of 9-NC should be investigated further as a chemotherapeutic strategy for breast cancer.

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